STUDIES

FROM

THE ROCKEFELLER INSTITUTE

FOR MEDICAL RESEARCH

REPRINTS

VOLUME XVI

NEW YORK

THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

capsule (4.8 by 1.6 cm.),[7] to which it adheres. With a hot wire a hole is burned through the cap into the glass neck. The open end of the body of the capsule is momentarily dipped just beneath the surface of very hot water, applied to the cap, and thus sealed in with an overlap of about 2 mm. Slight suction with the lips discloses whether the joint is tight. If not, a drop of hot water is touched to the leak. After drying, this framework is ready for the first dip in collodion.

Collodion solutions of two different consistencies are required, one for reinforcement, the other for the permeable membrane. After some experiment Squibb's collodion U. S. P. IX was found satisfactory, though a 4 per cent collodion, as a basis, may be made up from the formula, absolute alcohol 25 parts, ether 75 parts, pyroxylin 4 parts. Squibb's 4 per cent collodion or this solution of pyroxylin is too thin to give a proper membrane with one dip, but higher percentages of pyroxylin do not readily dissolve, and it is found most convenient to obtain the thicker solutions by evaporation. This is accomplished under vacuum with the application of gentle heat (water bath). The collodion boils rapidly, and evaporation is measured by the loss in volume.

The 12 per cent collodion required for the permeable membrane and the 14 to 15 per cent collodion required for reinforcement are obtained by evaporating 4 per cent collodion to one-third or less of its original volume. For evaporating and for dipping, museum jars 20 by 6.5 cm.,[8] with clamped tops and rubber gaskets, are used.

Of the entire surface of the gelatin capsule, the body is to be covered by the permeable membrane of the sac, and the cap by the heavier impermeable wall of collodion which serves to strengthen and support the membrane. This impermeable part is made first by dipping the inverted gelatin capsule (held by wedging the somewhat conical body into the end of a test-tube) neck downward into the 15 per cent collodion to a depth that just covers the joint between body and cap.[9] The capsule is dipped and withdrawn slowly to avoid air bubbles in

[7] Parke, Davis and Company, Detroit, Mich., Empty Capsules No. 12. A capsule of any suitable size may be used.

[8] Whitall Tatum and Company, Philadelphia, Pa.

[9] The open end of the glass neck may be covered with a bit of adhesive plaster.

the angle between neck and shoulder and to allow excess collodion to run down the neck.   After a few moments the collodion sets and ceases to flow, and the capsule, still held in the test-tube mouth, is inverted and set aside to dry.   Before drying is complete the excess collodion is cut away from the glass neck with a knife, leaving a collar 7 mm. high that effectually joins sac and neck together when it dries. This heavy, dry collodion membrane seals and reinforces the joints between body and cap and between cap and glass neck.

The sacs may be identified by means of small numbered paper labels sealed into the wall.   Tiny rectangles of very thin paper are numbered with India ink.   These are cut apart, curved slightly on a blotter with the rounded end of a test-tube, dipped with forceps into thin collodion and applied to the dry membrane just below the shoulder, where they adhere and become a permanent part of the wall (Text-fig. 1).

The capsules are now ready for the final dip.   They are to be handled, after the sacs are made, in 120 cc. (4 ounce) wide mouth bottles with rubber stoppers.   These stoppers have one hole centrally placed for the glass neck, and a second peripherally, which equalizes pressure in the bottle and the sac.   On account of its greater elasticity and resistance to autoclaving, a length of stethoscope tubing makes a good core for the central hole in the rubber stopper.   The hole is cut with a cork borer slightly smaller than the tubing, which is seized with forceps, pulled through with a projection of 1 cm. on the upper side of the stopper, and cut off flush with the bottom.   The use of the projection will be described later.

The neck of the reinforced capsule is inserted into the rubber stopper.   The capsule is then slowly lowered into 12 per cent collodion in the jar with a rotary to and fro motion which reduces the incidence of air bubbles.   It is immersed to the neck in the collodion, so that the joints at neck and body are reinforced.   On withdrawal, the coated capsule is suspended above the collodion by means of a slotted card slipped under the stopper and is allowed to drain a definite length of time, as will be described.   The membrane dries very little during this draining because of the ether-alcohol tension above the collodion. If a drop hangs from the capsule at the end of the drainage time it is removed by touching it to the surface of the collodion solution.   The
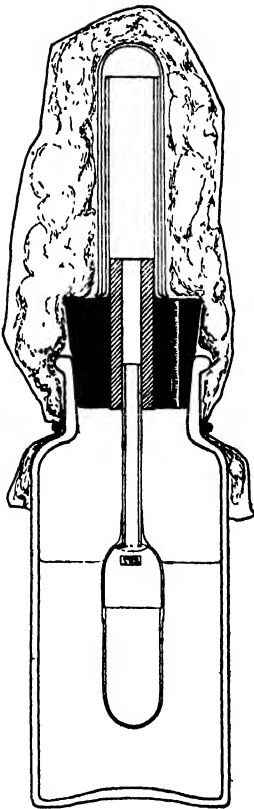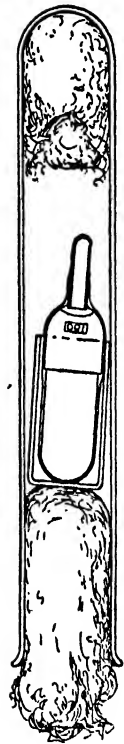
TEXT-FIG. 1.   TEXT-FIG. 2.   TEXT-FIG. 3.

TEXT-FIG. 1. Cross-section of a completed collodion sac in its protecting bottle. The various steps in the preparation of the sac are explained in the text.

TEXT-FIG. 2. A Pasteur pipette prepared for dry sterilization.

TEXT-FIG. 3. A sac in its cup and test-tube, after the neck has been sealed off in a small blast flame.

capsule is rapidly withdrawn from the jar and inverted to dry in the air for 1 to 2 minutes. It is then lowered into 95 per cent alcohol in a 4 ounce wide mouth bottle, where the collodion membrane finally sets.

The capsule with its collodion coating may remain in the alcohol indefinitely, but a few minutes are sufficient to extract the remaining ether and saturate the membrane with 95 per cent alcohol. As the viscosity of the collodion and the drainage determine the thickness of the sac, so the drying and the alcohol treatment determine its permeability.

After treatment in 95 per cent alcohol the capsule is plunged into cold water until the alcohol is removed and the gelatin capsule framework softens, a matter of an hour or less. The gelatin is then thoroughly washed out with a stream of hot water. For this purpose we have used a board with holes to accommodate twelve stoppers, resting on a basin of hot water. From the faucet, distributing tubes, ending in glass capillaries which are just long enough to reach into the sacs but not to pierce their bottoms, carry in the hot water and so wash the dissolved gelatin out through the neck. An hour or so of washing leaves the completed sac, attached to its glass neck by the collar of heavy collodion. It is then emptied with a Pasteur pipette and tested for strength and imperfections. Each sac must withstand an internal pressure of at least 25 cm. of mercury (5 pounds per square inch). The empty sac is immersed in distilled water in a 4 ounce bottle, and the second hole in the stopper is connected by a stub of glass tubing with a vacuum pump and gauge. The air in the bottle is exhausted 25 cm. A stream of air bubbles from the surface of the sac reveals any leak. If bubbles in the collodion were carefully avoided a leak is rare. Imperfect sacs are not worth repairing and are discarded.

The sac is then filled to the neck with distilled water and is ready for the autoclave. The expansion of water on autoclaving is sometimes sufficient to cause an overflow, and consequent loss of water on cooling. The projection of the stethoscope tubing above the stopper is therefore fitted with a short length of glass tubing open at the top. This forms an expansion chamber, from which the water drains back into the sac on cooling. To prevent contamination the glass tube is capped by a short, loosely fitting, inverted test-tube.

The top of the bottle is swathed in raw cotton and covered with paper, tied on. In this way contamination of either the inside or outside of the sac is avoided, and the sac is protected indefinitely for future use. Ten or twelve sacs are thus prepared at one time and then autoclaved at 15 pounds pressure for 30 minutes (Text-fig. 1).

Mode of Use.

Before discussing permeability, which is the vital factor in the collodion sac technique, the mode of inoculation and peritoneal implantation may be described briefly.

The most convenient instrument for filling and emptying the sacs and preserving their contents is a Pasteur pipette. Those which we use are made in quantities in the laboratory from 24 cm. lengths of rather heavy glass tubing pulled out in the middle and sealed off to make two pipettes. A bulb about 2 cm. in diameter is then blown just above the shoulder. The requisites are very hot glass and gentle pressure. The tip is cut just to reach the bottom of a Noguchi culture tube (20 by 1.5 cm.), and a wrapping of cotton below the bulb acts as a stopper for the tube, in which the pipette is dry steril-ized (Text-fig. 2). With these pipettes the sacs are filled, and after incubation the sac contents are similarly withdrawn and transferred to the test-tube which has protected the pipette from contamination. Then the pipette with its cotton collar serves as a plug for the tube and is used for subsequent withdrawal of fluid for examination.

Inoculation of small quantities of material into media in the sacs is accomplished with a platinum loop or a small sterile Wright pipette plugged with cotton. Such a pipette, flamed, and bent into a V saves the use of a Pasteur pipette in emptying out the distilled water from the sterilized sac preparatory to use. The bottle is inverted, the small arm of the pipette inserted upward through the neck, and gentle breath pressure expels the distilled water without danger of air contamination.

After being filled to the neck with a suitable medium and inoculated with bacteria or suspected material, the sac is withdrawn from the protecting bottle and the neck sealed off smoothly in a small blast flame. During this process and subsequently, contamination of the outside of the sac is avoided by carrying it in a short glass cup with

a flat bottom so that it will stand upright. Those used in the present work were made from 6 dram homeopathic vials cut off with a hot wire and a plunge into cold water. They are wider than the sacs and about 3 cm. long, so that the shoulder and neck project. The cups are sterilized by dry heat in wide test-tubes. When the neck of the sac is to be sealed off, one hand holds a cup containing the sac, while the other hand manipulates the rubber stopper. The cup, with its sac, is reinserted into the inverted test-tube until the neck has cooled (Text-fig. 3). The neck is then painted with a layer of collodion, which fuses with the collodion collar and so prevents the possibility of leakage between collar and neck. After a few minutes drying the sac is ready for intraperitoneal insertion. This is done aseptically under full ether anesthesia through a short incision in the abdominal wall above the umbilicus. The surgical technique need not be described, except to say that the incision should be sewed up firmly in layers. Metal skin clips save time. A dressing of cotton and collodion is sufficient. No bandage is required.

As many as eight such sacs have been tolerated by an adult rabbit without any apparent discomfort. The tissue reactions which often occur around the sacs will be discussed later. After an appropriate incubation period the rabbit is killed by an occipital blow and the sacs are recovered through a wide abdominal incision. They are again dropped into the glass cups, opened at the shoulder with a hot wire loop, and their contents transferred to a Noguchi tube by means of its Pasteur pipette.

General Characteristics of the Sacs.

Sacs made as just described have a capacity before autoclaving of from 6.5 to 7 cc. The upper third is a tough, impermeable wall which supports the glass neck and the permeable bottom. The lower two-thirds, with a surface area of about 14 sq. cm., is a thinner, moist, elastic membrane of high permeability. It is heavy enough, however, to stand immersion in water without collapsing and to withstand an internal pressure of 5 pounds per square inch. Such sacs rarely break during incubation. The walls of the sac are colorless and transparent.

Autoclaving at 15 pounds pressure for 30 minutes causes a uniform shrinkage to a capacity of 4.7 to 4.2 cc., corresponding to a diminution in surface area of approximately 25 to 30 per cent. Permeability is somewhat diminished but remains high. The shape and transparency of the sac are unaltered. The sacs may be kept sterile in distilled water in their bottles for months without appreciable loss in permeability, but the walls tend to become more brittle and inelastic.

Permeability.

The permeability of the membranes has been tested in a number of ways. The quantitative test by which variations in materials and methods of manufacture were compared consisted in dialyzing a 2 M solution of sodium chloride within the sac against 20 or 25 volumes of distilled water and titrating a sample of the dialysate from time to time with 0.02 M silver nitrate solution, sodium chromate being used as indicator. The quantitative relations were such· that when equilibrium was reached 1 cc. of the dialysate would precipitate 5 cc. (or 4 cc.) of the silver solution as silver chloride.

In 1915 Brown[10] contributed a careful study of collodion membranes and showed in particular that the permeability of membranes could be accurately controlled by complete drying, followed by immersion in ethyl alcohol of a definite dilution. Alcohol dilutions below 30 per cent confer practically no permeability on an air-dried membrane. 95 per cent alcohol produces a high degree of permeability. In preliminary experiments Brown's results were fully confirmed, and the value of his discovery was appreciated. His membranes, however, were prepared for chemical dialysis and did not require sterilization. On trial it was found that air-dried membranes, rendered permeable by alcohol treatment, lost their permeability again upon sterilization by heat. On the other hand, sacs which were not allowed to dry before immersion in alcohol lost little in permeability by sterilization in the Arnold sterilizer or the autoclave. Since highly permeable sacs were desired, 95 per cent alcohol was used as a routine.

Viscosity of the Collodion.—The percentage of pyroxylin in the collodion determines its viscosity, and viscosity in turn determines

[10] Brown, W., *Biochem. J.*, 1915, ix, 591.

the thickness of the collodion coating that will adhere to the dipped capsule. On account of rapid evaporation of the solvents the solution must be tested frequently and maintained at approximately the proper density. Commercial collodions vary considerably in density, and it is not a safe rule to evaporate a so called 4 per cent or U. S. P. collodion to one-third of its volume to obtain a 12 per cent solution.

The percentage of pyroxylin in the solution may be measured directly by weighing a specimen before and after evaporation to dryness. But this is not a convenient method, except as a check and to standardize a simple viscosimeter by which the thick collodions may be readily tested and compared.

The viscosimeter which we use consists merely of a 15 cm. length of Pyrex glass tubing, with square-cut ends, the internal diameter of which happens to be 0.365 cm. Near the middle of this tube file marks measure a distance of 5 cm. The tube is dipped into the collodion to be tested, a column is drawn up by suction well beyond the upper file mark, and the tube is withdrawn. As the column is then released in the vertical tube a stop-watch is used to time the meniscus over the measured distance of 5 cm. 12 per cent collodion at 20°C. requires 15± seconds to flow past the marks on the viscosimeter used.

Having standardized such a simple instrument it is easy to maintain thick collodion at the proper density by frequent tests and the addition of ether and absolute alcohol 3:1 to compensate for evaporation.

An even simpler method of determining viscosity, and one which is probably sufficiently accurate for the purpose, is to time the flow of collodion from a dipped capsule until the stream breaks and is succeeded by a series of drops. This time interval increases rapidly with the density of the collodion. In one test, for example, it increased from 12 seconds with a 10 per cent collodion to 1 minute, 15 seconds with the same collodion evaporated to approximately 12.7 per cent. The stream should flow about 1 minute before breaking.

The heavier collodion used for the neck and shoulder of the sac need not be standardized. We use it so thick that it will just flow smoothly, with no tendency to "jell." Collodion that has been used for permeable membranes until it has lost its clear transparency will serve the purpose.

Determination of the Draining Time.—The length of time that drainage is allowed to proceed influences the thickness of the collodion

coating and the evenness of its distribution over the surface of the capsule. Provided the first few drops are permitted to fall after the stream of collodion has broken, little is gained by prolonging the drainage. As will be shown later, slight variations in thickness are of minor importance, and sufficient uniformity is obtained by allowing a definite number of drops to form and fall.

Experiment 1.—From five sacs, after dipping, an unbroken stream of collodion flowed for an average of 1 minute, 16 seconds. Successive drops then fell after 1 minute, 26 seconds; 1 minute, 52 seconds; 2 minutes, 32 seconds; and 3 minutes, 29 seconds on the average. After 2 drops had fallen, 40 seconds were required to reduce the thickness of the membrane by the amount of 1 drop of collodion, and 57 seconds were required for the following drop to form.

It is hardly profitable to prolong the drainage time beyond the separation of the first few drops.

Effect of the Drying Time on Permeability.—

Experiment 2.—Five collodion sacs were made on No. 12 gelatin capsules by dipping once in 12 per cent collodion, draining 2 minutes, and drying for various intervals, as shown in Table I, before immersion in 95 per cent alcohol for 1 hour

TABLE I.

Sac No.	Drying time.	Sterilization.	Capacity.	
			Before sterilization.	After sterilization.
			cc.	*cc.*
81	0	Arnold sterilizer, 3 hrs.	6.4	4.6
76	15 sec.	Autoclave, 30 min.	6.2	4.8
82	30 "	Arnold sterilizer, 3 hrs.	6.4	4.7
80	1 min.	Autoclave, 30 min.	6.1	4.6
73	1 hr.*	Arnold sterilizer, 3 hrs.	7 3	6.2

* Completely dry in 1 hour.

The permeability of the sacs to sodium chloride was determined both before and after sterilization and is shown in Text-fig. 4. Sodium chloride diffused much more rapidly through the sacs which were not completely air-dried before the alcohol treatment. Sterilization, whether in the Arnold sterilizer at 100°C. or in the autoclave at 15 pounds pressure, did not greatly impair the permeability, notwithstanding the shrinkage which accompanied the process. The air-dried sac,

No. 73, on the other hand, shrank less during sterilization, but its permeability was so far lost in the Arnold sterilizer that no measurable amount of sodium chloride had passed through it in 6 hours, and only 1.30 units (on a scale of 5) had · passed in 48 hours.
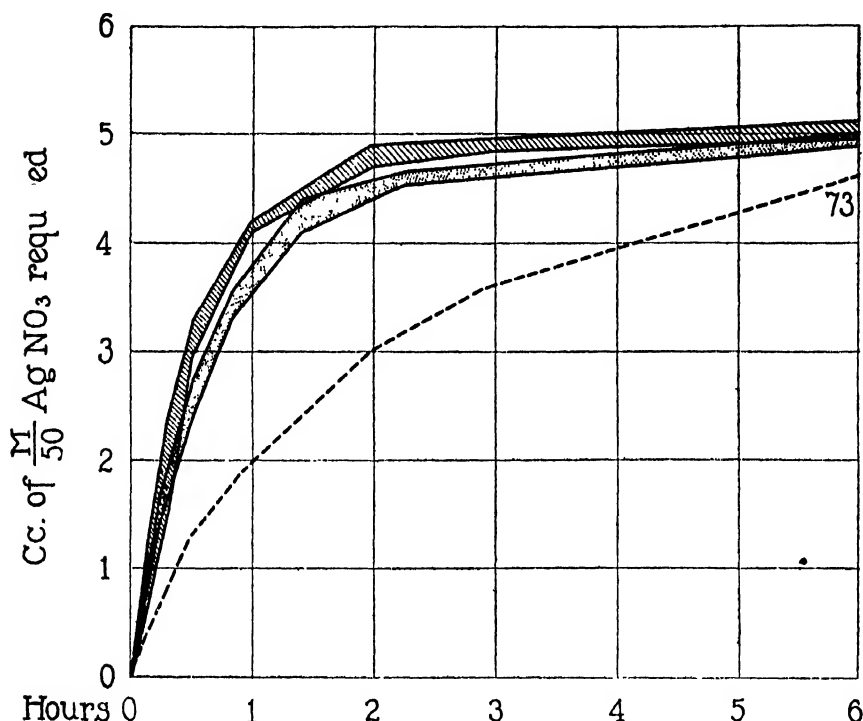
TEXT-FIG. 4. Experiment 2. The cross-hatched area covers variations in permeability of Sacs 81, 76, 82, and 80 (Table I) before sterilization. The stippled area covers variations in permeability of these sacs after sterilization. The broken line is the permeability curve of Sac 73, dried before the alcohol treatment. Sac 73, after sterilization, passed no measurable amount of sodium chloride in 6 hours.

Experiment 3.—In a similar experiment four sacs were dipped once, drained 3 minutes, dried 1, 2, 3, and 4 minutes respectively, and treated with 95 per cent alcohol over night. Their capacities before sterilization were 6.3, 6.1, 6.0, and 6.5 cc., and they shrank to 4.9, 4.7, 4.7, and 5.3 cc. in the autoclave. Their permeability before and after autoclaving is shown in Text-fig. 5.

From these two experiments it is evident that variations in the drying time have little influence on permeability, provided the membrane is still moist when immersed in alcohol. Sacs dried for 30

seconds to 1 minute are the most satisfactory. If plunged immediately into alcohol after drainage they tend to wrinkle and to show a smoky bluish opacity, and they are not so tough and strong as the partly air-dried sacs.
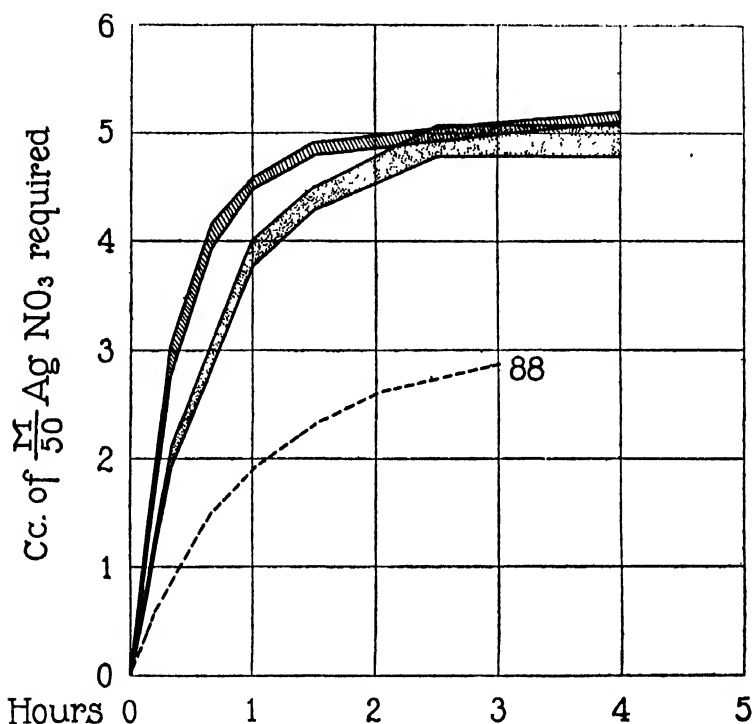

TEXT-FIG. 5. Experiment 3. The cross-hatched area covers variations in permeability of four sacs before sterilization. The stippled area covers variations in permeability of the same sacs after sterilization. The broken line is the permeability curve of Sac 88, not treated with alcohol before sterilization.

Importance of the Alcohol Treatment.—Other experiments indicate that treatment in 95 per cent alcohol for as short a time as 1 minute is sufficient to insure sacs of high permeability. It is only necessary that the alcohol should replace the ether-alcohol solvent of the collodion before the membrane is set by immersion in water. The importance of the alcohol treatment is illustrated in Text-fig. 5 by the permeability curve of Sac 88, which was drained 1.5 minutes, dried 30 seconds, and then plunged into water without the preliminary

immersion in alcohol. Although this sac was made of the same collodion as the others and shrank only from 6.9 cc. to 6.5 cc. during sterilization, ts permeability to sodium chloride was much less than that of alcohol-treated sacs.

Shrinkage of the Sacs during Sterilization.—The hot water used to wash out the gelatin framework of the sacs causes an initial loss in capacity from 7+ cc. to about 6.6 cc. Sterilization by heat further shrinks the membrane to an average capacity of 4.5 cc. The loss in capacity and permeability due to heating is absolute and does not continue once the limit is reached.

Experiment 4. Effect of Re-Autoclaving.—Four sacs, dipped once in 12 per cent collodion, drained 1.5 minutes, dried 30 seconds, and immersed in 95 per cent alcohol for 1 hour, were autoclaved at 15 pounds pressure for 30 minutes. Two of the sacs were then re-autoclaved under similar conditions. The capacity of the first two sacs was 4.5 and 5.1 cc. respectively, of the second two 4.4 and 5.05 cc. All four sacs had a practically identical permeability as shown by Text-fig. 6. The curve incidentally illustrates the uniformity of collodion sacs made as described above.
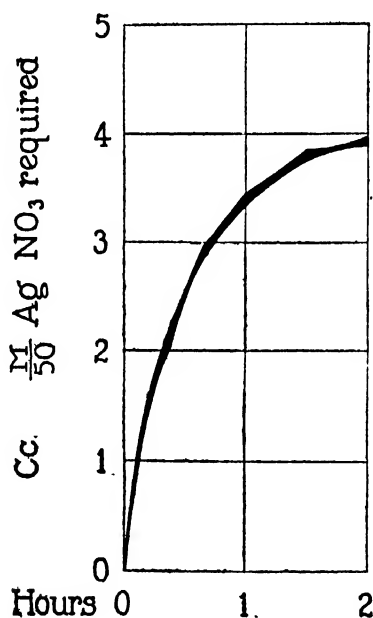
TEXT-FIG. 6. Experiment 4. The solid black area covers variations in permeability of four sacs, two of which were autoclaved once, the other two autoclaved twice.

The relation between the diminution in capacity and in surface area of the sacs on sterilization is shown in the following experiment.

Experiment 5.—Eight sacs made in an identical manner from the same collodion solution were divided into two groups of four each. After volume measurements had been taken, four of the sacs were autoclaved and their capacities again determined. All the sacs were then cut open so that they could be flattened out, shadowgraphs were made on photographic paper, and the areas measured with a planimeter. The average measurements are shown in Table II, in which are included similar figures for two sacs that were completely dried before the alcohol treatment.

TABLE II.

Condition and No. of sacs.	Volume.				Surface			
	Before autoclaving.		After autoclaving.		Before autoclaving.		After autoclaving	
	Body.	Total.	Body.	Total	Body.	Total.	Body.	Total.
	cc.	cc.	cc.	cc.	sq cm.	sq. cm.	sq. cm.	sq cm
Two dried sacs.			4.9	6.95			14.3	21.9
Four undried sacs; not autoclaved.	4 5	6 72			14.0	21.6		
Four undried sacs; autoclaved.	4 45	6.67	2.72	4 5			9.6	15.8
Capacity loss.			39 per cent.	33 per cent.			31 per cent.	27 per cent.

It is seen from Table II that sterilization of the permeable sacs caused a loss of 33 per cent in capacity, corresponding to a surface shrinkage of 27 per cent. The losses were not proportionately distributed between the impermeable cap and the permeable body, which lost 39 per cent in capacity and 31 per cent in surface area.

This shrinkage on heating is due largely to the alcohol treatment of undried sacs. Dried sacs, even though treated subsequently with alcohol, shrink but little in the alcohol, as Brown[10] has shown, or in the autoclave (Table II). Undried sacs, not treated with alcohol, also shrink but little on sterilization. The part played by shrinkage in decreasing permeability has probably been overestimated. That the shrinkage itself is of minor importance is shown by a comparison

of the permeability of alcohol-treated undried sacs with that of sacs prepared by other methods.

In this connection it may be pointed out that titrations with sodium chloride do not give complete information with regard to the permeability of the sac walls. The sodium chloride molecules and their constituent atoms are of relatively small size, and the rate at which they diffuse through the permeable membranes suggests that the pores of the membrane must be several times the size of the molecules to permit such rapid passage. The volume shrinkage on sterilization is approximately 33 per cent, which corresponds to a diminution in permeable surface area of 31 per cent. Assuming that the pores diminish correspondingly, it is conceivable that while small molecules might still pass through with ease, other molecules which more nearly approached the unshrunken pores in size might now be entirely held back. So far, however, among the substances tested none has been found which passes through the unheated, but not through the heated membranes.

Relation of Thickness to Permeability.—Another factor emphasized in permeability experiments with collodion membranes is thickness. In the present method, within limits, this factor also is found to be secondary in importance to the alcohol treatment. The viscosity of the collodion solution and the drainage and drying time are the variables in determining thickness and have been experimentally controlled (Experiments 1, 2, and 3). A 12 per cent solution forms a sac of sufficient strength for intraperitoneal incubation. Thicker sacs, however, may be used without great loss in permeability.

Experiment 6.—Three collodion sacs were made on a gelatin capsule framework. No. 141 was dipped once in 12 per cent collodion, No. 142 twice, and No. 143 three times. The sacs were drained 2 minutes and dried 45 seconds between each dip. All were immersed in 95 per cent alcohol for 1 hour, washed, and autoclaved. Sac 141 was thin and transparent. Sac 142 was of medium thickness, with a slightly smoky opacity. Sac 143 was very thick and tough, with a smoky opacity. Capacities before autoclaving, 6.8, 6.1, and 6.4 cc. respectively; after autoclaving, 4.7, 4.0, and 4.2 cc. Such sacs in their thinnest area, just below the joint, were found to measure 0.09, 0.31, and 0.52 mm. in thickness after sterilization.

The results of permeability tests with 4 cc. of 2 M sodium chloride against 96 cc. of distilled water are shown in Text-fig. 7.
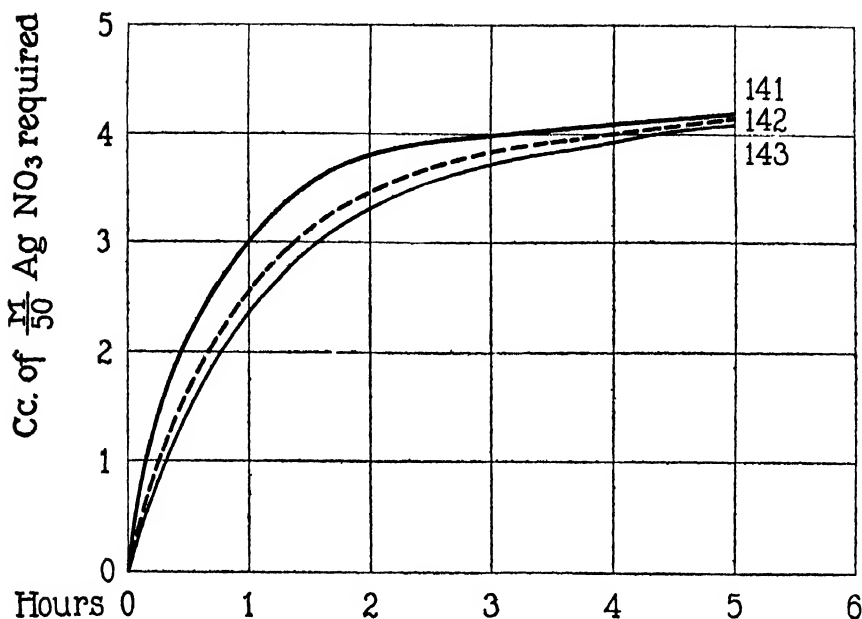
TEXT-FIG. 7. Experiment 6. The permeability curves, after sterilization, of three sacs of widely different thicknesses. Sac 141 dipped once in 12 per cent collodion, Sac 142 dipped twice, and Sac 143 dipped three times. In this experiment equilibrium in dialysis is represented by 4 cc. of silver nitrate. The error in the final readings is due to the removal of successive portions of dialysate for test. This loss in volume was not compensated during the experiment.

The difference between the sacs is less than would be expected from the comparative thickness of their walls and indicates that slight variations in thickness would be of no practical significance.

Qualitative Tests of Permeability with Other Substances.—In addition to the titrations of permeability with sodium chloride a number of qualitative tests with other substances have been made. These tests were incidental to other experiments and are therefore incomplete, but the results are useful in indicating the relative permeability of the sacs.

1. Various inorganic salts passed through the sacs with an ease approaching that of sodium chloride. With such substances the endosmotic pressure of a concentrated solution is so quickly lowered by passage through the sac that the level of liquid within the sac is hardly raised significantly before equilibrium is established.

2. Oxygen in solution rapidly diffuses through the sac wall and restores the color to methylene blue reduced by the action of dextrose broth. This fact probably explains the consistent failure of representative strict anaerobes to grow in permeable sacs intraperitoneally implanted. Haggard and Henderson[11] estimate the oxygen tension of the peritoneal cavity at about 45 mm. So far no method has been devised by which this handicap may be overcome.

3. During intraperitoneal incubation the hydrogen ion concentration of sac contents tends to come to an equilibrium with that of the peritoneal fluid. Ascitic fluid, dilute rabbit serum, and broth, for example, whatever their initial reaction, and $\frac{M}{15}$ phosphate mixtures, in proportions to give various pH concentrations, have all been reduced to a pH of 7.4 to 7.5. *In vitro* it is found that the primary and secondary phosphates diffuse through the walls with equal facility. The dialysate comes almost immediately to the pH concentration of the phosphate mixture used.

4. Among the simpler organic compounds, dextrose alone has been tested. It soon shows its presence on the opposite side of the membrane, but diffuses more slowly than do inorganic salts and so gives opportunity for a considerable endosmosis before equilibrium is established.

5. Nutrient materials of meat infusion broth pass through the sacs with sufficient rapidity to promote a luxuriant growth of bacteria (*B. typhosus, B. pyocyaneus*) inoculated into distilled water. The straw-colored pigments of the broth are likewise diffusible. Nutrient materials are obtainable from body fluids in a similar manner. Heavy growths of *B. pyocyaneus, B. typhosus*, and Type I pneumococcus were obtained by incubating inoculated sacs of distilled water over night in a rabbit. *B. pfeifferi*, lacking hemoglobin, did not grow under similar conditions.

6. The diffusible products of bacterial metabolism have not been determined by analysis. That the sacs are permeable to these products is shown by the tissue reactions which occur around actively growing cultures. The sacs themselves are practically inert, and it is common to find uninoculated control sacs lying free among the intestines without any observable irritation of the surrounding tissues. Sacs which contain living bacteria, on the other hand, are usually the center of an active proliferative process. They are found wrapped in folds of thickened and injected omentum or among intestinal adhesions, sections of which show fibrin deposits, accumulations of leucocytes, rarely to the extent of pus formation, localized hemorrhages, infiltration with fibroblasts, and newly formed capillaries; in short, the various elements and stages of a degenerative and regenerative tissue reaction.

7. No evidence has been observed that hemoglobin or other unsplit proteins will pass through the sacs. In two experiments in which highly potent antimeningococcic serum was surrounded by a suspension of meningococci no agglutination occurred.

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[11] Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1919, xxxviii, 71.

In summary, it may be said that the sacs as described are permeable to gases in solution, to inorganic salts, to dextrose, to certain protein-split products which are nutritive to bacteria, and to certain toxic products of bacterial metabolism, but they hold back antibodies, unsplit proteins, and formed elements such as bacteria and body cells.

SUMMARY.

A standardized method is described in detail by which collodion sacs suitable for intraperitoneal incubation and for other bacteriological experiments may be produced in large numbers, sterilized, and handled with convenience and the minimum danger of contamination. Various factors influencing permeability have been subjected to experiment. Like Brown, we found that immersion in alcohol is the most important factor, but the high permeability conferred by alcohol treatment is lost during heat sterilization if the membrane was previously allowed to dry. Quantitative experiments on the dialysis of sodium chloride, and simple tests with other substances indicate the general character of the membranes and their probable field of usefulness in bacteriology.

A MECHANICAL MEASURING INSTRUMENT FOR STERILE LIQUIDS.

BY FREDERICK L. GATES, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Part of the strain and tedium of immunological research is connected with the distribution of identical portions of a given liquid among a large number of tubes. Ordinarily this task is performed by means of a graduated pipette, in which the movement of the liquid is controlled by the ball of the finger and regulated by watching the meniscus as it passes the graduation marks. The process is time-consuming, and the precise control necessary to stop the meniscus exactly on the mark is a tax on eye and hand.

The instrument here described[1] was first devised as a substitute for the graduated pipette in immunological tests. Since it mechanically measures any amount of liquid within its capacity in a sterile manner it may find application also in serum and vaccine laboratories, in media preparation rooms, and wherever aliquot portions of a liquid are to be measured out.

As with the graduated pipette, the principle involved is the displacement of the required amount of liquid by a similar amount of air at atmospheric pressure. The air is measured by a graduated syringe, which may be set to deliver any amount within its capacity. Before coming in contact with the liquid, the air is twice filtered through raw cotton plugs. Provided the container is sterile all danger of contamination is eliminated.

Description of the Instrument.

The syringe *F*, of any required capacity, is screwed into one outlet of a three-way stop-cock *H*, which also supports a rigid hollow sleeve

[1] The instrument may be obtained from the Central Scientific Company, 460 East Ohio Street, Chicago, Ill.

G and forms the frame of the instrument. This sleeve G guides an inner sliding sleeve E which operates the key of the stop-cock through the slotted arm I and is itself operated by friction contact with the sliding rod B. This rod engages the plunger rod C of the syringe, and by means of the adjustable lock-nut L limits its travel. The finger, slipped through the ring A, raises and lowers the plunger rod and operates the stop-cock mechanism. The plunger is surmounted by a slotted sleeve D in which the plunger rod fits loosely and operates the plunger only toward the end of the stroke in either direction. The object of these sliding mechanisms is to turn the key of the stop-cock before the plunger moves.

In Text-fig. 4 the instrument is pictured at the end of the down-stroke. The stop-cock is open in the direction indicated in Text-fig. 2. A complete up- and downstroke may be followed in detail. As the finger, slipped through A, raises the plunger rod C, the rod B is raised also, carrying with it, by friction contact, the sleeve E. This sleeve in turn operates the key of the stop-cock through the slotted arm I so that during the travel of E the key turns from position 2 (Text-fig. 2) to position 1 (Text-fig. 1). During this first part of the upstroke the plunger does not move, since the plunger rod slides freely in the loose sleeve D. When the stop on C reaches the end of the slot in D, the sleeve E also reaches the frame G, and the key of the stop-cock reaches position 1. The stop-cock is now open for the admission of air to the syringe barrel through the side outlet of the cock and the short rubber tubing K and cotton filter N.

As the finger continues to raise the plunger rod the plunger is now raised also by the engagement of the stop on the rod in the end of the slot in D. The rod B slides through sleeve E until its travel is stopped by contact of the lock-nut L with sleeve E, which is already in contact with G. The plunger has now risen in the barrel a definite distance, determined by the position of the lock-nut L on the rod B, and a measured quantity of air at atmospheric pressure is contained within the syringe.

On the downstroke the first movement is again that of A, B, E, and I, operating to turn the key of the stop-cock back to position 2. This accomplished, the plunger rod C engages the sleeve D at the bottom of the slot and drives the plunger down. The air in the syringe

TEXT-FIGS. 1, 2, and 3. Various positions of the stop-cock key. (1) Position for the admission of air to the syringe; (2) for the expulsion of air into the liquid container; (3) for filling the container by suction.

TEXT-FIG. 4. The complete instrument. The lettering is explained in the text.

barrel is expelled into the container O attached to the heavy rubber tubing M, and displaces a corresponding quantity of liquid which is thus delivered by the apparatus.

The instrument may be set to deliver any volume of air, and consequently any volume of liquid, within the capacity of the syringe. To set it for a given volume the lock-nut L is loosened and the plunger raised exactly to the desired graduation mark on the syringe barrel. Then the lock-nut is set snugly against the sleeve E, in contact with G, and tightened.

Any sort of container with an inlet for the measured air and an outlet for the liquid may be used. The only requirement is that the system shall be air-tight except at the intake and outlet. The instrument may be attached to it directly or through a length of tubing. The elasticity or rigidity of the walls is of no consequence, since the system returns to atmospheric pressure after each delivery.

In immunological tests in which relatively small amounts of liquids are handled, a 5 or 10 cc. pipette may be used. The instrument is operated with one hand, and the pipette held and directed with the other. Larger amounts of liquid may be contained in flasks or bottles with a tightly fitting two-hole rubber stopper and a delivery tube reaching to the bottom. In such instances the instrument may be clamped in a frame and the rod B operated by a pedal, leaving both hands free.

When a pipette is used it is refilled as follows: The plunger is raised to the top of the stroke, and the split thumb-nut J partly unscrewed. It first releases the slotted arm I, and then, by friction, it turns the key of the stop-cock from position 1 to position 3 (Text-fig. 3). The stop-cock is now open from the side outlet to the pipette, which is filled by suction on N. The thumb-nut is again tightened, returning the key to position 1 and locking the arm I in place.

In order to minimize errors in measurement, attention should be given to several precautions in the use of the apparatus. (1) For exact measurements the syringe must be accurately calibrated and the lock-nut carefully set to bring the plunger exactly to the mark. (2) The system must be air-tight. Since the instrument itself need not be sterilized, the stop-cock and plunger may be lubricated with a heavy oil or grease. The addition of a little beeswax to the plunger

lubricant keeps the plunger in position at the top of the stroke until it is forced down by contact of the plunger rod. (3) An error is introduced by a difference in level between the surface of the liquid in the container and the point of outlet. Usually this error is negligible. A difference in level of 10 cm. causes an error of about 1 per cent. (4) Care must be taken that the air is not expelled too rapidly into the container so as to force the liquid out under pressure, lest more than the required amount be delivered by its own momentum. This is recognizable by the subsequent retreat of the liquid from the delivery tip, as equilibrium is reestablished. A complete stroke should be up, and then down, for each delivery. A small delivery tip lessens the error due to the adherence of the last drop of liquid. (5) When air and liquid are at different temperatures an error is introduced by expansion or contraction of the measured air on contact with the liquid. For accuracy, therefore, the air and liquid should be at the same temperature. Practically, when the instrument is handled with reasonable care, these sources of error are not encountered.

The facility with which sterile solutions may be measured out without contamination should be emphasized. The liquid does not pass through the instrument, and nothing comes in contact with it but twice filtered air.

SUMMARY.

An instrument is described by which aliquot portions of a liquid may be mechanically measured and delivered. It was devised especially for use in immunological experiments, to take the place of a graduated pipette in setting up serum tests. The instrument may be set for any quantity within its capacity and measures sterile liquids without danger of contamination. It may therefore find a wider application in other procedures requiring sterile measurements of small amounts of liquid.

FACTORS INFLUENCING ANAEROBIOSIS, WITH SPECIAL REFERENCE TO THE USE OF FRESH TISSUE.

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PLATE 2.

Anaerobic cultivation of bacteria has in recent years developed into an important field of bacteriological research. Anaerobic methods have been extensively applied, for example, to the study of the bacterial flora of war wounds and to the investigation of diseases of unknown origin.

In these investigations the cultivation of anaerobes in tubes containing bits of fresh tissue has been extensively employed. Although study of some of the principles involved has led to improvements in method, a method for the quantitative examination of the factors involved is lacking and the establishment of practical rules and indications to meet the requirements of the more fastidious of this group of microorganisms is still to be attained. We shall attempt to show the influence of certain elements in promoting or in hindering the development of strict anaerobic conditions in culture tubes and so to indicate more exactly the value of these factors in anaerobic cultivation.

Aside from the use of mechanical methods for removing oxygen from ordinary media, much attention has been devoted in recent years to the cultivation of anaerobes in a fluid or semisolid medium containing a fragment of fresh sterile tissue, usually kidney, and overlaid with a layer of paraffin oil. The value of the tissue fragment was pointed out by Theobald Smith[1] in 1899, and rediscovered by Tarozzi[2] and Wrzosek[3] in 1905. The use of paraffin oil is credited to Legros.[4]

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[1] Smith, T., *J. Boston Soc. Med. Sc.*, 1898–99, iii, 340.

[2] Tarozzi, G., *Centr. Bakt., 1te Abt., Orig.*, 1905, xxxviii, 619.

[3] Wrzosek, A., *Wien. klin. Woch.*, 1905, xviii, 1268.

[4] Legros, G., Recherches bactériologiques sur les gangrènes gazeuses aiguës, Paris, 1902; *Compt. rend. Soc. biol.*, 1902, liv, 1337.

In 1911 Noguchi,[5] using tall columns of serum water overlaid with paraffin oil in narrow tubes and containing a fragment of fresh tissue, was able to grow *Spiro-chæta pallida* under strictly anaerobic conditions.   He did not rely on the kidney tissue or the paraffin oil for the production of anaerobiosis, but for the first time employed with these a combination of hydrogen gas, vacuum, and pyrogallic acid in an anaerobic jar.

Later, in the cultivation of the globoid bodies of poliomyelitis[6] it was found that the mechanical precautions could sometimes be omitted.

Following these reports the tissue-serum water or ascitic fluid tubes overlaid with paraffin oil came to be widely used as an anaerobic method and some investigators, seeking strictly anaerobic conditions, have disregarded the elaborate precautions which Noguchi employed. But the successful use of the tissue method alone, which appears to be simple, has been found to require considerable patience and experience, and the method has often suffered for lack of quantitative standardization and through misunderstanding of underlying principles.

A number of variables are involved.   The first requisite for a study of these variables is a delicate and precise reversible indicator for the presence or absence of free oxygen in solution.   The indicator should react in the presence of culture media, so that it may be added directly to the materials to be tested, and should not interfere with the reducing activity of other components of the medium or arrest the growth of test organisms.   Methylene blue fulfills these requirements in a satisfactory manner.

Theobald Smith[7] in 1896 reported the reduction of methylene blue and other indicators in the closed area of fermentation tubes by sterile peptone broth. The presence of muscle or grape sugar and the application of heat increased the speed of the reaction. Peptone and dextrose water were inert. Spina[8] had already noted the reduction of methylene blue by nutrient gelatin, but not by agar.

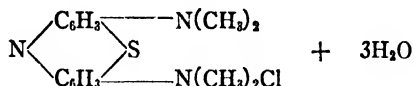[5] Noguchi, H., *J. Exp. Med.*, 1911, xiv, 99.

[6] Flexner, S., and Noguchi, H., *J. Exp. Med.*, 1913, xviii, 461.

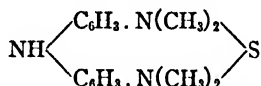[7] Smith, T., *Centr. Bakt., 1te Abt.*, 1896, xix, 181.

[8] Spina, A., *Centr. Bakt.*, 1887, ii, 71.

Methylene Blue as Indicator.

By the action of weak deoxidizing agents methylene blue (tetramethylthionine chloride)

$$N \Big\langle {}^{C_6H_3 \text{---} N(CH_3)_2}_{C_6H_3 \text{---} N(CH_3)_2Cl} \Big\rangle S \quad + \quad 3H_2O$$

is converted readily to colorless leucomethylene blue, or α-(p)-tetramethyldiamino-thiodiphenylamine

$$NH \Big\langle {}^{C_6H_3 . N(CH_3)_2}_{C_6H_3 . N(CH_3)_2} \Big\rangle S$$

The reaction is reversible, and the conversions proceed rapidly at incubator temperature in the presence of free oxygen or under the attack of the deoxidizing agent after the free oxygen has been consumed.

Experiments were made to determine the sensitiveness of the indicator and the conditions under which it reacts. Ordinarily 0.1 cc. of a 1 per cent aqueous solution of methylene blue in 10 cc. of 2 per cent dextrose peptone broth in a test-tube is placed in the anaerobic jar and the removal of free oxygen is indicated by the gradual decolorization of the dye. Since methylene blue is not decolorized under similar conditions in a medium of distilled water, we sought the ingredient in the dextrose broth which promotes the decolorization.

Relation of the pH of the Medium to Its Reducing Activity.—Beef infusion, 2 per cent peptone solution, and 2 per cent dextrose, with methylene blue, when boiled in a water bath to remove the air were not decolorized. Nor were mixtures of any two, or of all three of the ingredients. But so far in this experiment the hydrogen ion concentration of these mixtures had not been considered. The foregoing materials were retested in a solution of 0.01 M sodium hydroxide. Decolorization was immediate on heating to drive off the air, but the dye was resolved into simpler elements than the leuco form, thus destroying its value as an indicator.

2 per cent dextrose solution, colored with methylene blue, was mixed with an equal volume of buffer phosphate mixtures in $\frac{M}{15}$ solution, to produce a range of pH concentrations from 6.6 to 7.8. Tubes of these mixtures were heated to expel the air and to increase the

velocity of reaction.   The solutions of a pH of 7.4, 7.6, and 7.8 were quickly decolorized.   On cooling and exposure to air the blue color as promptly returned.   Similar but less rapid effects were obtained with the 2 per cent peptone solution.   The beef infusion appeared to be inert.

The reducing action of dextrose in alkaline solution is well known as the basis of the Fehling and Benedict tests in urine analysis.   The same reaction is utilized here.   The routine use of methylene blue in dextrose peptone broth is simply a convenient method of assembling the materials in a weakly alkaline solution.

The delicacy of the reaction depends upon the feeble reducing power of the medium at the chosen hydrogen ion concentration and at incubator temperature.   An equilibrium is established between the rate of diffusion of oxygen through the medium and the activity of the reducing agent.   It is only when the access of oxygen is practically completely inhibited that the weakly alkaline dextrose solution is able to fix the remaining traces of the gas and then to attack the methylene blue.

Comparison of Different Amounts of Methylene Blue.—From 0.1 to 1 cc. of a 1 per cent aqueous solution of methylene blue in 10 cc. of dextrose broth was decolorized with practically equal rapidity in less than 24 hours in a McIntosh and Fildes jar.[9]   For the purposes of this study, variations in the small quantity of the dye used in the medium were of little significance.

Effect of Temperature.—Like other chemical reactions, the rate of reduction is a function of the temperature.   When access of free oxygen is prevented decolorization does not occur in 24 hours at 4°C.   It proceeds slowly at room temperature (21°C.) and rapidly at 37°C. and higher temperatures.

Estimation of the State of the Medium.—It has been noted above that the state of the indicator, whether blue or colorless, depends upon the relative rate of the admission and diffusion of oxygen from the air and the activity of a reducing substance, *e.g.* dextrose, in the medium.   The equilibrium between these two forces may be observed in a narrow test-tube by the depth below the surface at which the colorless zone begins.   While methylene blue may not be decolorized in an oxygen-free liquid in the absence of a reducing substance, decolorization indicates a strictly anaerobic condition, and the return of the color is an index of the return of oxygen to the medium.

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[9] McIntosh, J., and Fildes, P., *Lancet*, 1916, i, 768.

With these indications of the sensitiveness of the reaction and of the factors that control its progress, we proceeded with a study of the various elements in the technique under investigation.

Comparison of Liquid Paraffin Oil and Solid Vaseline as a Seal.

The tissue medium technique as generally employed involves the use of liquid paraffin oil as a seal or supernatant to the medium. It was presumed that the oil, besides preventing evaporation, favored deoxidation by opposing a barrier to the air. Rosenthal[10] suggested the use of lanolin (melting point 42°C.) as a seal, and recently Fildes[11] has stated that oil has practically no effect in preventing the return of oxygen to the medium, while solid paraffins merely delay its passage. In view of the importance of an effective seal, a quantitative comparison of oil and vaseline seemed advisable.

Experiment 1.—Twelve tubes, 1.5 by 20 cm., each containing one-sixteenth of the kidney of a 1,600 gm. rabbit, were filled with 1 per cent dextrose broth to a height of 9.5 cm.; 2 drops of sterile 0.5 per cent aqueous methylene blue were added as indicator. Series A consisted of four tubes, not sealed (air control). Series B, four tubes overlaid with 2.5 cc. of paraffin oil. Series C, four tubes overlaid with 2.5 cc. of vaseline. All incubated at 37.5°C.

When observed after 16½ hours the tubes of Series A were decolorized 2 cm. from the bottom of the tube. Tubes of Series B decolorized 3 cm. from bottom. Tubes of Series C completely decolorized.

When observed after 42 hours, the tubes of Series A and B showed an increase of 1 cm. in the height of the decolorized column. Tubes of Series C remained completely decolorized. There was no further increase in the height of the decolorized columns in the tubes of Series A and B.

Repetition with smaller fragments of kidney yielded similar results.

As shown by the point at which a balance was established between the access and diffusion of oxygen and the reducing action of the kidney tissue, the addition of paraffin oil produced only a slight increase in the length of the anaerobic column, the final ratio of the oil-covered to the air-covered tubes being 4:3.

[10] Rosenthal, G., *Compt. rend. Soc. biol.*, 1902, liii, 1132.

[11] Fildes, P., *Med. Research Com., Nat. Health Insurance, Special Rep. Series*, No. 12, 1917, 59.

Experiment 2.—Sixteen 20 by 1.5 cm. tubes, filled with broth and methylene blue as before, but without kidney tissue. Series A, four tubes not sealed. Series B, eight tubes sealed with paraffin oil in a column from 0.5 to 4 cm. in height. Series C, four tubes, vaseline seal 1 cm. high. All were placed in a McIntosh and Fildes jar,[9] the oxygen was removed by combustion, and the tubes were incubated at 37.5°C.

After 18 hours Series A was completely decolorized. Series B, 0.5 to 1 cc. of oil, completely decolorized (1.5 to 2.5 cc. decolorized in 42 hours, 3 to 4 cc. in 60 hours). Series C, completely decolorized. Removal from jar and exposure to air caused a prompt return of color in the tubes of Series A and B. Series C remained colorless permanently.

The results of Experiments 1 and 2 are illustrated in Fig. 1.

In the McIntosh and Fildes jar a slight reduction in pressure occurs as a result of the combination of oxygen and hydrogen. The partial vacuum tends mechanically to lower the tension of oxygen dissolved in the medium and so hastens the establishment of strict anaerobic conditions through the action of a reducing agent. When large amounts of paraffin oil are employed, this favorable action is retarded. Indeed, the oil seems to serve as a reservoir for oxygen in solution. Except to prevent evaporation and to maintain anaerobic conditions after removal, the use of any seal appears to be unnecessary in a properly manipulated McIntosh and Fildes jar.

Experiment 3.—Six 20 by 1.5 cm. tubes containing 10 cc. of dextrose broth were colored with 1 drop of 1 per cent methylene blue. Series A, two tubes, unsealed. Series B, two tubes, overlaid with 4 cc. of paraffin oil. Series C, two tubes, overlaid with 2.5 cc. of vaseline. All tubes heated in a water bath until color disappeared, then plunged into cool water to bring to room temperature and to solidify the vaseline.

The blue color appeared immediately at the surface of the unsealed tubes and at the surface of the medium in contact with the oil. Streaks of blue carried by convection currents descended to the bottom of the tubes, there to be slowly decolorized again. After 20 minutes a zone of blue was established to a depth of 3.5 cm. in the unsealed tubes and to 3 cm. in the oil-covered tubes. The vaseline-covered tubes remained colorless.

After standing for 72 hours at room temperature the blue zone had descended to a depth of 4.7 cm. in the unsealed tubes and to 4.6 cm. in the oil-sealed tubes. During the following days the unsealed and the oil-sealed tubes came to an equilibrium approximately 6 cm. below the surface of the medium. The vaseline-sealed tubes remained colorless throughout.

Under air or paraffin oil, dextrose broth is unable alone to over-come the diffusion of oxygen, which is hardly retarded by the paraffin oil seal. In the depths of the tube, however, the combined reducing action of broth and kidney is able to establish and maintain an oxygen-free zone, which is slightly higher in the paraffin oil-covered tubes. Under vaseline, on the other hand, owing solely to its solid state at incubator temperature, the access of oxygen is prevented, and the dextrose and kidney tissue in the course of a few hours fix all the oxygen remaining in solution. Other aids to deoxygenation, such as a McIntosh and Fildes jar or the action of heat in reducing the oxygen solubility of the medium and increasing the chemical activity of the alkaline dextrose solution, exert a similar action in hastening the establishment of anaerobic conditions in a culture tube, provided that access of atmospheric oxygen is prevented by an impervious solid seal such as vaseline.

We conclude that the paraffin oil seal extensively used in anaerobic culture work and in gas analysis is practically valueless except to prevent evaporation. The seal itself may contain enough oxygen in solution to defeat the very object which it is used to attain. A layer of vaseline, on the other hand, is an oxygen-resisting seal that materi-ally aids the action of deoxidizing agents in the medium.[12]

Standardization of the Kidney Tissue Component.

Four functions have been ascribed to the fragment of kidney utilized in the tissue technique: a reducing activity, the formation of a nidus, the contribution of nutritive elements to the medium, and an effect upon the pH concentration by acid production. That the kidney tissue component is an active reducing agent has been well known since 1885 through the researches of Ehrlich and others and is demonstrated anew in Experiment 1 of this communication. But the zone of its action in the culture tube has not been subjected to quantitative estimation.

[12] Hard, inelastic paraffin waxes may be less useful through rupture of their contact with the glass by changes of temperature in the tubes.

Smith,[1] who was the first to advocate the use of tissue fragments, gave no indication of the size to be used. Tarozzi[2] advocated a cube of about 1 cm. Noguchi[13] suggested that the kidney of an adult rabbit should be cut into about ten pieces, each approximately the size of a split chestnut. Other original workers have not defined the tissue component so carefully and the tendency in general has been to use too small a piece, especially for primary cultivations and early transplants.

Comparison of the Reducing Effect of Different Amounts of Kidney Tissue.—We tested the reducing effect of pieces of various sizes upon a medium containing methylene blue.

Experiment 4.—Two sets of three 20 by 1.5 cm. tubes were prepared, each containing 10 cc. of aerated 1 per cent dextrose broth and 1 drop of 1 per cent aqueous methylene blue in addition to the kidney fragments. The fragments were cut as uniformly as possible from 5 mm. cross-sections through the middle of the kidneys from an 1,800 gm. rabbit. In Set 1 a single piece of kidney was used. Tube A contained one-eighth, Tube B one-fourth, and Tube C one-half of a 5 mm. cross-section. In Set 2 the pieces were cut into eighths of the original cross-section. Tubes A, B, and C were the same as the corresponding tubes of Set 1, except that Tube B contained two-eighths and Tube C four-eighths of a cross-section in two and four separate fragments. No seal. Incubated at 37.5°C.

In Tubes B and C of each series decolorization began in a few moments around the kidney tissue at the bottom of the tube. Later results are shown in Table I and are illustrated in Fig. 2.

After 42 hours the tubes were removed from the incubator and allowed to stand at room temperature. After 20 hours at about 21°C. the level of decolorization had dropped 0.5 to 1 cm. in each tube.

TABLE I.

Comparison of the Reducing Effect of Different Amounts of Kidney Tissue.

Set No.	Duration of incubation.	Height of decolorized column from bottom of tube.		
		Tube A; small fragment of kidney.	Tube B; twice as much as Tube A.	Tube C; four times as much as Tube A.
	hrs.	*cm.*	*cm.*	*cm.*
1	16	0.4	2.3	3.0
2	16	1.0	1.8	3.0
1	42	1.7	3.5	4.4
2	42	1.8	3.4	4.4

[13] Noguchi, H., *Münch. med. Woch.*, 1912, lix, 1937.

Different amounts of kidney tissue have different reducing effects. The larger the amount, the greater is the reduction. Small pieces are of little value for producing an anaerobic zone. Very large pieces do not produce a proportionately large zone of reduction. With 0.6 to 0.8 gm. of kidney tissue a zone sufficient for practical purposes is obtained.[14] The tissue may be used in one fragment or in several. The balance between the penetration of oxygen from the surface into the medium and the reducing activity of the tissue occurs at a level determined by the temperature, other things being equal. The higher the temperature, up to 37°C., the less is the oxygen solubility and the greater the reduction.

Nature of the Reducing Substances in the Kidney Tissue.—Kidney tissue in unsealed tubes such as those of Experiment 4 exposed to the air at room temperature will maintain an anaerobic zone at the bottom of the tube for a period of weeks or months. It is not conceivable that the reduction is dependent on the maintenance of the activity of living cells. The question arises then, as to the nature of the substance responsible for the reduction. In considering this question, which properly belongs to physiology and biochemistry, we were soon led away from the subject in hand. Preliminary experiments indicated that the activity still resided in filtered extracts of kidney tissue, and that the reducing substance concerned is relatively heat-stable, so that boiled, or even autoclaved kidney is not entirely without effect, but other considerations make it seem unprofitable to attempt to modify the use of fragments of fresh sterile kidney tissue in this anaerobic technique. Thunberg[15] refers to former researches on the reducing activity of tissues and reports his evidence of the enzymotic nature of the reaction.

Before the reducing activity of fresh tissue in anaerobic cultivation was generally recognized, it was suggested that the tissue fragment might act in an obscure and passive manner as a focus for bacterial multiplication. The demonstration of a favorable reaction on the

[14] The kidneys of a medium sized rabbit, 1,400 to 1,700 gm., weigh about 6 to 6.5 gm. Eight to ten fragments may be obtained from each kidney. The large kidneys of full grown rabbits yield twelve to sixteen fragments of proper size.

[15] Thunberg, T., *Skandin. Arch. Physiol.*, 1918, xxxv, 163.

surrounding medium made such an assumption unnecessary. Some investigators, however, acting on the earlier hypothesis, have advocated the use of small pieces of inorganic substances, asbestos wool or iron tacks, for example,[16] as a nidus for anaerobic growth.

Our experiments with these substances in the presence of methylene blue need not be elaborated here. They indicate that washed asbestos wool has no effect in promoting an anaerobic zone. Indeed, in a feeble reducing medium the decolorization of the methylene blue was somewhat retarded. The oxidation of iron tacks, on the other hand, soon reduced the dye in their vicinity. It seems clear that the production of an anaerobic condition depends upon a chemical reaction rather than upon the presence of inert material.

Nature of the Culture Medium.

Presence of a Reducing Substance.—Methylene blue serves as an indicator of the presence or absence of free oxygen only in the presence of a reducing substance. For this reason it is not an accurate index for the removal of oxygen by physical means. Our observations led us to conclude, however, that complete deoxygenation is only very slowly accomplished by the diffusion of oxygen in solution into an oxygen-free atmosphere such as is provided by the method of Buchner[17] or that of McIntosh and Fildes.[9]

In a recent communication Barber[18] indicates that some strict anaerobes may be destroyed even by short exposures—less than an hour—to atmospheric oxygen. Under certain conditions, therefore, it may be important to attain strictly anaerobic conditions in the culture tube in the shortest possible time after inoculation or else to inoculate an already deoxygenated medium.[19] Among the aids to such a procedure is the addition of an active reducing agent (kidney tissue, dextrose, peptone) to the culture medium.

Culture media in general may be divided into two classes, those which contain an active reducing substance, and those which are prac-

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[16] Douglas, S. R., Fleming, A., and Colebrook, L., *Lancet*, 1917, ii, 530.

[17] Buchner, H., *Centr. Bakt.*, 1888, iv, 149.

[18] Barber, M. A., *J. Exp. Med.*, 1920, xxxii, 295.

[19] Tarozzi[2] and Noguchi[5] undoubtedly obtained such a condition by preliminary incubation of tissue media to insure sterility.

tically inert. Artificial media containing dextrose or peptone belong in the first category. Ascitic fluid and dilute serum, widely used in anaerobic culture work, belong in the second class. Although small amounts of a copper-reducing substance may be demonstrated in these liquids, their deoxygenating action is relatively slight, and hardly to be considered of practical value. Thus, while kidney tissue was able to decolorize 10 cc. of ascitic fluid containing methylene blue under a vaseline seal in 10 days, similar tubes of ascitic fluid, without kidney tissue, still retained a pale blue color after 6 weeks observation.

The efficacy of dextrose as a reducing agent in alkaline solution suggested its addition to the ascitic fluid medium. Combinations of 0.1 to 2 per cent of dextrose were made by the addition of 10 per cent dextrose solution in isotonic saline solution to the ascitic fluid. Vaseline-sealed tubes containing ascitic fluid and methylene blue and 0.1 per cent dextrose were almost decolorized in 7 days. The larger amounts of dextrose up to 2 per cent, with the aid of a McIntosh and Fildes jar, decolorized the ascitic fluid in 5 to 6 days. Dextrose peptone broth was even more efficacious. One part of 1 per cent dextrose peptone broth with two parts of ascitic fluid decolorized methylene blue under a vaseline seal in less than 42 hours.

In the absence of a reducing agent tubes of ascitic fluid from which air is rigidly excluded may not become decolorized over a period of weeks. The addition of small amounts of dextrose, or the presence of kidney tissue renders ascitic fluid medium oxygen-free in a relatively short time. It should be understood that dextrose here is being considered solely as a reducing agent. Other effects—change of pH concentration through bacterial action, gas formation, etc.— may make its addition undesirable in certain instances.

Physical State of the Medium.—A second character of culture medium which influences the establishment of anaerobic conditions is its physical state. Fluid media suffer the disadvantages of ready diffusion of oxygen throughout the tube, of the development of convection currents on even slight changes of temperature, and of the transfer of oxygen to the depths of the tube by any agitation. These phenomena are easily observed in air or oil-covered tubes showing a decolorized zone at the bottom.

Semisolid media, formed by the addition of small amounts of agar, are not subject to these influences to the same extent. It is not surprising, therefore, to find that anaerobic conditions are more readily maintained in the depths of a semisolid culture tube. Thus, the effect of different amounts of agar on the diffusion of oxygen through dextrose broth was tested.

Experiment 5.—Tubes were filled with 1 per cent dextrose peptone broth and 2 per cent dextrose agar in proportions to give percentages of agar from 0 to 0.5 in volumes of 10 cc. The mixtures were colored with methylene blue and heated in the water bath to complete decolorization. They were then plunged into water to solidify the agar and incubated at 37°C. The diffusion of oxygen from the surface downward was measured in all the tubes by the advance of the returning color. After 5 and 24 hours the blue zones had descended as shown in Table II, where they remained during subsequent observations.

TABLE II.

Penetration of Oxygen into Semisolid Medium.

Tube No.	Amount of agar.	Penetration of oxygen from surface of medium.		
		After 5 hrs.	After 24 hrs.	After 48 hrs.
	per cent	*cm.*	*cm.*	*cm.*
1	0	1.0	4.0	Complete.
2	0.02	1.0	1.5	3.3
3	0.04	0.9	1.2	2.2
4	0.06	0.9	1.1	1.3
5	0.1	0.8	1.0	1.1
6	0.2	0.8	1.0	1.1
7	0.5	0.7	0.9	1.0

Even so small an amount as 0.02 per cent of agar may inhibit the diffusion of oxygen to the depths of a culture tube, or at least so retard it that dextrose broth is able to maintain anaerobic conditions below a certain level. This level occurred at 1 cm. from the surface when 0.5 per cent of agar was employed.

A more striking experiment is afforded by a comparison of the reducing effect of kidney tissue in peptone broth and in the same broth made semisolid by the addition of 0.25 per cent agar.

Experiment 6.—Series A; tubes contained a 9 cm. column of plain broth. Series B; the same plus kidney tissue. Series C; tubes contained a 9 cm. column of semi-solid medium (peptone broth plus 0.25 per cent agar). Series D; the same plus kidney tissue. All were colored with methylene blue and were incubated unsealed at 37.5°C. Table III shows the height of the colorless zones which developed from the bottom of the tube in each instance.

TABLE III.

Development of Anaerobic Conditions by the Action of Kidney Tissue in Fluid and Semisolid Medium.

Medium.	Zone of decolorization measured from bottom of tube.		
	After 1 day.	After 2 days.	After 6 days.
	cm.	*cm.*	*cm.*
Series A; plain broth without kidney tissue............	0	0	0
" B; " " with kidney tissue	1.5	2.0	1.4
" C; semisolid medium without kidney tissue.......	7.4	7.3	7.3
" D; " " with kidney tissue.........	7.0	7.3	7.3

Besides showing that oxygen penetrates slowly into semisolid media this experiment demonstrates the reducing power of peptone (Series C). Comparison of Series C with Series D suggests that the reducing power of kidney tissue in a semisolid medium is confined to the zone surrounding the tissue, since its effect did not extend further than that of the peptone broth alone.

This observation is confirmed by the following experiment with a medium of much less reducing power, so that the kidney tissue alone was responsible for the production of an anaerobic zone.

Experiment 7.—Series A, two tubes, each containing one-tenth of a medium sized kidney, 7.5 cc. of dilute rabbit serum, 2.5 cc. of 2 per cent plain agar, and 1 drop of methylene blue, making a 0.5 per cent semisolid medium. Series B, two tubes, each containing the same ingredients, except that 2.5 cc. of plain broth were substituted for the agar, making a fluid control. Control tubes of each medium, without kidney tissue, were also set up. Vaseline seals. Tubes incubated at 37.5°C. for 24 hours. The tubes of Series A showed a decolorized zone 1.7 and 1.7 cm. high respectively, sharply demarcated from the deep blue agar above. Control, without kidney, deep blue. The tubes of Series B were decolorized 2.4 and 2.3 cm. from the bottom, respectively, shading off gradually to a deep blue above. Control, without kidney, deep blue. After 48 hours the semisolid tubes showed a clear-cut colorless zone, 2.7 and 2.6 cm. high; agar deep blue above. Control

deep blue. The fluid tubes showed a colorless zone approximately 4 to 4.5 cm. high, shading off so gradually that its limits were difficult to determine. The fluid above and the control were light blue.

From these experiments a semisolid medium is seen to favor the establishment of anaerobic conditions by the exclusion of oxygen from the depths of the tube. Unless a reducing agent is diffused through the medium, however, this advantage is offset by a restriction of the reducing power of the kidney tissue to the immediate vicinity of the tissue fragment. These observations may explain in part the difficulty often encountered in growing strict anaerobes in semisolid medium, especially in initial cultivation.

Length of the Column of Medium as an Aid in Deoxygenation.—It has become the standard practice in anaerobic cultivation by the tissue method to use 12 to 15 cc. of fluid, or a column 10 to 12 cm. in height in a long narrow culture tube 20 by 1.5 cm. Anaerobiosis in the depths of the tube would seem to be favored by the restricted surface exposed under paraffin oil and by the length of the column of liquid.

It was found in the present study that in a reducing medium such as dextrose broth a column length of from 8 to 16 cm., overlaid with paraffin oil, favors the decolorization of methylene blue, which occurs first at the bottom of the tube and gradually extends toward the surface. Under vaseline, on the other hand, columns of different lengths decolorize with equal rapidity. In dextrose broth the reducing action proceeds throughout the tube. When it is more localized, as with kidney tissue in a less active medium (plain broth), the effect is somewhat different.

Experiment 8.—Three sets of tubes of plain broth colored with methylene blue, each containing one-sixteenth of a kidney of a large rabbit, the broth columns being 2, 4, 6, 8, 10, and 12 cm. long. Series A, unsealed; Series B, overlaid with 2 cm. of oil; Series C, overlaid with 1 cm. of vaseline. Control tubes of broth without kidney, column 8 cm. long, unsealed and also with oil and vaseline seals. Incubated at 37.5°C. In 16 hours decolorization had proceeded as shown in Table IV.

When oxygen is excluded (Series C) the fragment of kidney tissue is able to produce an anaerobic zone 2.1 cm. high in 16 hours. That

it did not do so in the 2, 4, and 6 cm. tubes of Series A and B under air or oil is due to the penetration of oxygen. A column of fluid 8, 10, or 12 cm. long maintained at a constant temperature without agitation practically serves as a seal for the lower levels. But the same object is accomplished by a vaseline seal with considerable saving in medium; 7 or 8 cm. of the medium suffice.

TABLE IV.

Effect of the Height of the Medium Column on the Anaerobic Zone Produced by Kidney Tissue.

Series.	Height of decolorized column from bottom of tube after 16 hrs.						Control.
	Length of fluid column.						
	2 cm.	4 cm.	6 cm.	8 cm.	10 cm.	12 cm.	8 cm.
	cm.	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	
A	1.0	1.5	1.8	2.2	2.1	2.1	Unchanged.
B	1.3	1.8	1.9	2.1	2.0	2.1	"
C	2.0	2.1	2.0	2.1	2.1	2.1	"

Effect of Reducing Agents on the pH of the Medium.

Although this study deals only with the deoxygenation of culture media, it is necessary to know whether the reducing agents employed have any effect upon other essential factors in anaerobic cultivation; for example, the hydrogen ion concentration of the medium. Accordingly, tubes were filled with ascitic fluid or with ascitic fluid and dextrose peptone broth, with or without kidney tissue. To each tube 5 drops of phenol red were added and the tubes incubated at 37.5°C. under a vaseline seal. When observed at intervals during the following days and weeks, the tubes without kidney tissue showed no change from the original hydrogen ion concentration of 8 +. On the other hand, the kidney tissue in 20 hours had produced a clear yellow zone approximately 1.5 cm. from the bottom of the tube. The effect was the same in ascitic fluid and in the mixture of ascitic fluid and broth. The next day and thereafter, all the tubes containing kidney tissue showed a gradation from purplish pink (pH 7.8 to 8) at the surface downward in the acid direction to a clear yellow in the middle and lower portions of the column of medium. This color

gradually became diffused throughout the tubes, bringing the medium to an orange-yellow comparable to pH 7.4 on the phenol red scale.

Ascitic fluid itself is usually alkaline, showing a pH 7.8 to 8+. This alkalinity is progressive if the fluid is allowed to stand exposed to the air but may be retarded by a layer of paraffin oil. From the foregoing experiment it is seen that the alkalinity, once established, remains unaltered for weeks under a vaseline seal. Dextrose and peptone do not in themselves produce acid and thus change the hydrogen ion concentrations. Only when they are split by bacterial activity do they have this effect. Kidney tissue produces acids; a large fragment (0.8 gm.) changes the hydrogen ion concentration in its immediate vicinity to about pH 7. This acid becomes gradually diffused throughout the medium and may ultimately bring the entire column of medium to a favorable hydrogen ion concentration (pH 7.4). The final concentration, then, depends on the ratio of the original alkalinity of the ascitic fluid to the acid production of the kidney.

SUMMARY AND CONCLUSIONS.

This study was undertaken with the object of determining the part played by the several component elements of the tissue method of anaerobic cultivation in the establishment of anaerobic conditions in the culture tube. Data have been presented to show the suitability of methylene blue as an indicator of reduction processes in culture media by which the removal of the last traces of oxygen may be demonstrated. With methylene blue as the indicator, the elements subjected to experiment included the choice of a seal for culture tubes, the activity and requisite size of the kidney tissue fragment, the chemical and physical characters of the medium which promote or retard deoxygenation, the length of the column of medium, and the advantages of external aids such as the McIntosh and Fildes anaerobic jar.

As a result of our experiments, we have come to the following conclusions:

1. Liquid paraffin oil, used extensively as a seal for anaerobic cultures and in gas analysis, has very little value in inhibiting the access of oxygen. Solid vaseline, on the other hand, forms an effective

oxygen-resisting seal. The difference is due to the physical states of the substances at incubator temperature.

2. Fresh kidney tissue is an active reducing agent and quickly decolorizes methylene blue in its vicinity. The reducing effect of fresh kidney tissue is relative to the amount used. As a reducing agent, at least 0.6 gm. per tube is required for the establishment of an adequate oxygen-free zone.

3. Culture media may be classified as reducing or non-reducing. Those containing dextrose or peptone in a faintly alkaline solution belong to the former class. Ascitic fluid and dilute serum belong to the latter class, for their content of reducing substances is practically insignificant. For the prompt establishment of strictly anaerobic conditions these media require the addition of reducing substances such as dextrose, peptone, or kidney tissue aided by an effective seal or an anaerobic jar.

4. Semisolid media effectively inhibit the penetration of oxygen to the depths of the tube, but they likewise limit the diffusion of reducing substances and presumably of nutrient substances from imbedded kidney tissue.

5. The length of the column of medium is of minor importance under a vaseline seal.

We clearly recognize the impracticability of standardizing a biological technique which by its very nature must be subject to wide modifications for special purposes. Such variations from a standard are especially necessary in the search for unknown organisms, and in work with hitherto uncultivated microbes in which the tissue technique has been successfully applied by Noguchi.

We wish, therefore, to present the results of our studies simply as guides in the variation and control of the elements examined and to make certain suggestions relative to the establishment of strictly anaerobic conditions in the culture tube. The numerous other factors of equal importance which must be taken into account—hydrogen ion concentration, source and character of nutritive elements, temperature, time, etc.—are outside the limited scope of the present report.

For the establishment of strictly anaerobic conditions in the culture tube, we would suggest (1) the substitution of solid vaseline for liquid paraffin oil as an oxygen-resisting seal; (2) the use of large pieces

of fresh kidney, the standard size to be upwards of 0.6 gm. unless other reducing substances are present in the medium; (3) the addition of peptone or dextrose or both in the form of peptone dextrose broth in fractional percentages to non-reducing media such as ascitic fluid or serum to aid in the prompt establishment of anaerobic conditions; (4) the use of the McIntosh and Fildes jar as a further aid to the prompt deoxygenation of the medium; (5) for reasons of economy the use of smaller amounts of culture medium, for example, 7 to 8 cc., under a vaseline seal; and (6) in dealing with anaerobes which may be injured by exposure to oxygen it might be advisable to prepare the medium a day or two in advance and to incubate it under a vaseline seal so that sterility is assured and the anaerobic conditions are already established when inoculation is made. The infected material is then introduced with a capillary pipette in the vicinity of the kidney tissue and the seal restored by gentle heating to melt a portion of the superposed vaseline.

EXPLANATION OF PLATE 2.

FIG. 1. A comparison of paraffin oil and vaseline as oxygen-resisting seals Sample tubes from Experiments 1 and 2. Dextrose broth and methylene blue without and with kidney tissue, unsealed and under paraffin oil and vaseline. Tubes 1, 3, and 5, without kidney, were deoxygenated by the dextrose broth in a McIntosh and Fildes jar. On removal from the jar the color has returned in the unsealed and oil-covered tubes (Nos. 1 and 3), denoting the penetration of oxygen into the medium. The vaseline-covered tube (No. 5) remains colorless.

Tubes 2, 4, and 6, with kidney, show its reducing effect in the depths of the tube. The unsealed broth (Tube 2) shows almost as extensive a zone of decolorization as the oil-covered broth (Tube 4). Aided by complete exclusion of oxygen, the kidney and dextrose broth have completed the deoxygenation of the vaseline-covered tube (No. 6). ·

FIG. 2. A comparison of the deoxidizing value of kidney tissue fragments of different size. A, a fragment of the size ordinarily employed. B, a fragment twice the size of A. C, a fragment four times the size of A. The establishment of an oxygen-free zone is denoted by the decolorization of the medium.
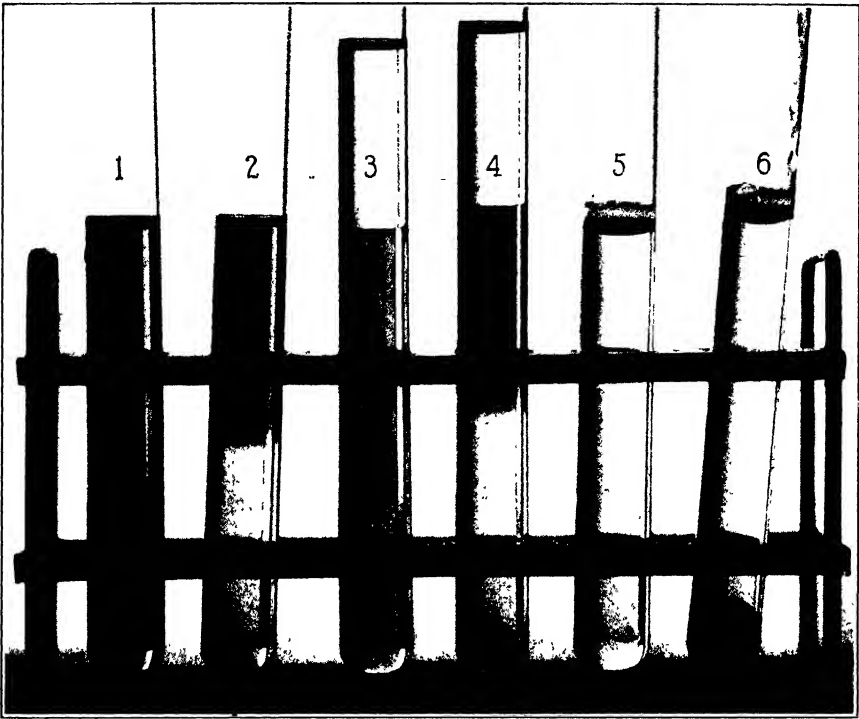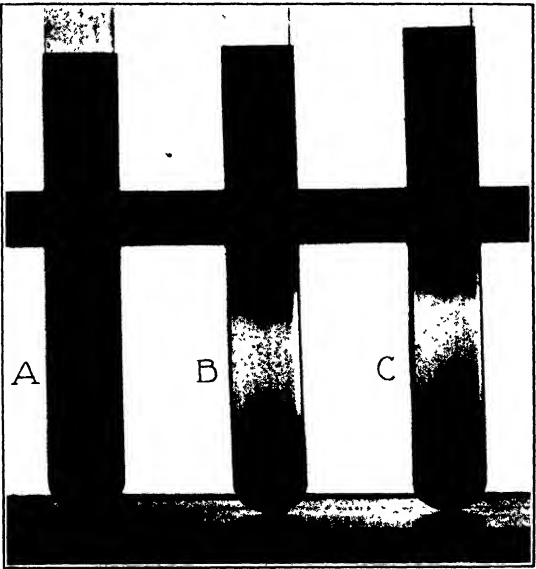
Fig. 1.

Fig. 2.

(Gates and Olitsky: Factors influencing anaerobiosis)

PROPHYLACTIC INOCULATION AGAINST YELLOW FEVER.

By HIDEYO NOGUCHI, M.D., and WENCESLAO PAREJA, M.D.

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Sufficient grounds for the possibility of establishing a prophylactic vaccination against yellow fever are furnished by the facts that (1) a person acquires a state of complete immunity enduring for a period of years after recovery from an attack of yellow fever, and (2) the serum of such a person, and also of guinea-pigs once inoculated with the blood of yellow fever patients, contains varying amounts of antibodies against *Leptospira icteroides*.[1] That such vaccination is a distinct possibility is shown in these experiments:

Vaccination in Guinea-Pigs.

In the first series of experiments, carried out in Ecuador, thirty guinea-pigs ranging in weight from 280 to 320 gm., and recently received from mountainous districts, were divided into three equal groups and inoculated subcutaneously with the killed culture of *Leptospira icteroides* (heated at 60°C. for fifteen minutes in a water bath) containing about 500,000 organisms per cubic centimeter, the first group receiving 1 c.c., the second 0.3 c.c., and the third, 0.1 c.c. All were kept under careful observation, daily records being made of the temperature and other reactions. Two weeks after inoculation they were tested for resistance to a virulent leptospira culture of the Guayaquil strain No. 1, applied to the scarified surface of the skin. As controls, eight uninoculated guinea-pigs of similar weights were given the same culture in the same manner and at the same time. The culture employed was 10 days old and killed guinea-pigs within eight days in a dose of 1 c.c. of a 1:10,000 dilution by intraperitoneal injection. The results are recorded in Table 1.

1. Noguchi, Hideyo. J. Exper. Med. 30: 1, 9 (July) 1919.

As the table shows, the injection of the vaccine produced neither febrile nor local reactions in the guinea-pig.

TABLE 1.

Results of Vaccination of Guinea-Pigs against Leptospira Icteroides.

Number of Guinea-Pig	Amount of Vaccine, C.c.	Local or Febrile Reactions	Subsequent Infection. After	Result of Infection	Pfeiffer Phenomenon After 4 weeks
601	1	None	14 days	Well	Positive
602	1	None	14 days	Well	Positive
604	1	None	14 days	Well	Positive
605	1	None	14 days	Well	Positive
607	1	None	14 days	Death in 10 days without jaundice	
609	1	None	14 days	Well	Not done
610	1	None	14 days	Well	Not done
611	0.3	None	14 days	Well	Positive
613	0 3	None	14 days	Well	Positive
614	0.3	None	14 days	Well	Positive
·615	0.3	None	14 days	Well	Positive
618	0.3	None	14 days	Well	Positive
620	0.3	None	14 days	Well	Not done
622	0.1	None	14 days	Death in 12 days; typical	
623	0.1	None	14 days	Well	Positive
624	0.1	None	14 days	Slight icterus after 9 days; recovered	Positive
625	0.1	None	14 days	Well	Positive
627	0.1	Ncne	14 days	Well	Positive
629	0 1	None	14 days	Well	Positive

Controls
631   Death in 9 days; symptoms and lesions typical
532   Death in 9 days; symptoms and lesions typical
633   Death in 11 days; symptoms and lesions typical
634   Death in 9 days; symptoms and lesions typical
635   Death in 13 days; symptoms and lesions typical
636   Death in 3 days through an intercurrent infection
637   Death in 10 days; symptoms and lesions typical
638   Death in 11 days; symptoms and lesions typical

By the use of a sufficient amount of killed culture of *Leptospira icteroides*, guinea-pigs were made resistant to a subsequent inoculation which invariably produced typical symptoms and lesions and death in normal guinea-pigs. A smaller amount (0.1 c.c. in the present

series) conferred imperfect protection, although four of six animals remained well, and one recovered after a mild attack of the infection.

Other series of experiments have been carried out by one of us (Noguchi) at The Rockefeller Institute with guinea-pigs of American stock. The results were similar to the foregoing and will be published in detail later.

Effects of Vaccination in Man.

The almost total absence of any constitutional or local reaction except the development of a subsequent definite immunity following the injection of a killed culture of Leptospira icteroides into guinea-pigs furnished a basis for applying this procedure to nonimmune persons for the purpose of protection. There is great need of a prophylactic measure in countries in which yellow fever still prevails and in which a nonimmune population may become exposed to the disease. The nonimmune population, of course, avoids visiting the yellow fever foci through dread of the disease, and the supply of labor and other commercial and industrial relations is thereby seriously affected. Yellow fever in such countries becomes an important national economic issue.

The trial of prophylactic vaccination against yellow fever in Ecuador was precipitated by the occasion of the national celebration that was to take place during the first part of October, 1918, in Gauyaquil. A battalion known as the "Vincedores," consisting chiefly of nonimmune soldiers from the highlands, was ordered to participate in the ceremony, and Governor Pareja of Guayaquil suggested the possibility of trying the anti-yellow fever vaccination on these soldiers. His suggestion was accepted by the military authorities and sanctioned by President Baquerizo. At the barracks in Guayaquil to which the battalion was ordered, approximately 300 soldiers are stationed regularly. During the period of six months from January to June, 1918, there had been fifty-eight cases of yellow fever with twenty-nine deaths in these barracks, which was an unusual outbreak. The introduction of sanitary measures (isolation of suspected cases by mosquito nets, the closing and frequent cleaning of drinking water tanks, etc.) had checked the epidemic, and only one officer died of yellow fever there between July and October, 1918.

A journey was made to Quito (9,000 feet above sea level), September 18, 1918, and on September 20, eleven persons were inoculated in order that we might observe the effect of the vaccination. The histories and physical examinations of these men were made by Dr. Pareja, who excluded any one who was suspected to be immune or had any sign of ill health. Samples of the blood were taken from the median basilic vein before vaccination for tests of immune bodies for the leptospira and for comparison with serum drawn from the same individuals after the vaccination.[2]

The vaccine consisted of a rich killed culture of the Guayaquil strain No. 1 containing about 2,000,000 leptospiras per cubic centimeter, heated in a water bath at 60°C. for fifteen minutes, and used ninety-six hours after killing; no antiseptics were added. The heated organisms were fragmented, tortuous and granular, the original forms being no longer recognizable; the liquid was slightly grayish, but limpid. Inoculations were made subcutaneously, usually on the left upper arm, and different doses were used, four persons receiving 1 c.c., four, 0.3 c.c., and three, 0.1 c.c.

The local reactions comprised a slight reddening (a few centimeters square, without induration) of the injected area, which persisted about twenty-four hours; in only one instance was it somewhat more extensive; it endured forty-eight hours, but was not more painful than might have been expected from the mechanical effect of the injection. Subjectively, some experienced a slight dulness in the head, but all slept well, and the appetite was unaffected. In one instance there were slight pains in the shoulders which disappeared within forty-eight hours. There were slight brachycardia and hypothermia, which began after about ten hours and lasted from twenty-four to forty-eight hours; in one instance the pulse rate went down to 50, and in several to 56. At the same time the temperature was about 1 to 2 degrees C. below normal. These objective effects were by no means proportional to the amount of vaccine injected. Within seventy-two hours all symptoms had disappeared.

A second inoculation of the same doses was made, Sept. 24, 1918, in three individuals of the 1 c.c. group, one of the 0.3 c.c. group, and two of the 0.1 c.c. group. No local or constitutional reactions followed. September 26, blood was drawn from all the inoculated persons for the purpose of determining the leptospiricidal properties of the serum.

The serums taken before and after vaccination were examined, September 30, for the Pfeiffer phenomenon. To 2 c.c. of the serum was added 1 c.c. of a rich, actively motile culture of the No. 1 strain, and the mixture was introduced into the peritoneal cavity of a normal guinea-pig. After from thirty minutes to one hour the fluid was pipetted from the peritoneal cavity and examined under the dark field microscope. The results of this examination in ten soldiers, and the subsequent fate of the animals used, are recorded in Table 2.

The serums of the ten soldiers taken previous to vaccination had no action on the organisms either in respect to the Pfeiffer tests or the subsequent course of the infection in the guinea-pigs injected with them, while the serums taken after vaccination gave a positive Pfeiffer reaction even when the amount of the killed culture inoculated had been as small as 0.1 c.c. The final infection, however, was not prevented in most instances, the animals receiving the serums from persons vaccinated with a single dose of 0.3 c.c. and single or double doses of 0.1 c.c. all developing typical symptoms and dying after a period involving delays of from two to four days, as compared with those receiving the normal serums. In the case of the four animals that received serum from soldiers inoculated twice with 1 c.c. and 0.3 c.c., however, there were three that survived. Hence it may be concluded that, given a sufficient amount of the killed culture, not only the Pfeiffer phenomenon but also the protection of the animal against the infection with a large amount of the virus may be brought about in human serum. The results from the serum of soldiers receiving smaller amounts of the killed culture, in which the animals finally succumbed to infection notwithstanding a positive Pfeiffer reaction, were to have been expected, in view of the enormous quantity of culture used in the tests; moreover, it does not follow that the soldiers yielding these serums were not protected against a natural leptospira infection, since under natural circumstances infection of

man is never induced by such large numbers of the organism.   Indeed, it may be assumed that the number of leptospiras introduced into the human body by the mosquito is small and probably readily taken care of in persons whose serum contains antibodies enough to give a positive Pfeiffer reaction.   It will, of course, be advisable to adopt double or triple injections of the vaccine for prophylactic inoculation, when possible.

September 24, 149 soldiers of the battalion mentioned were inoculated in Quito with the same vaccine, 120 being given 1 c.c. and the

TABLE 2.

Results of Vaccination against Leptospira Icteroides in Ten Human Beings.

Soldier No.	Dose C c.	Number of Vaccinations	Serum Taken Before Vaccination		Serum Taken After Vaccination	
			Pfeiffer Phenomenon	Fate of Animals	Pfeiffer Phenomenon	Fate of Animals
1	1	2	−	Death in 5 days	+	Survived
2	1	2	−	Death in 6 days	+	Death in 9 days
3	1	2	−	Death in 6 days	+	Survived
4	0.3	2	−	Death in 6 days	+	Survived
5	0.3	1	−	Death in 6 days	+	Death in 9 days
6	0.3	1	−	Death in 5 days	+	Death in 7 days
7	0.3	1	−	Death in 4 days	+	· Death in 8 days
8	0.1	2	−	Death in 6 days	+	Death in 8 days
9	0.1	1	−	Death in 5 days	+	Death in 7 days
10	0.1	1	−	Death in 6 days	+	Death in 8 days
Saline control ...	....		−	Death in 5 days		

remainder 2 c.c.   The same brief effects, namely, brachycardia and a drop in temperature, were noted.   In only one instance were marked headache, loss of appetite and muscular pains complained of.   In this officer a mild urticaria, which disappeared in about nine days, developed around the site of injection.   A boy soldier of 14 years had a pulse rate as low as 44 on the day following the vaccination, but had no subjective symptoms.

October 8, the day after their arrival at Guayaquil, the remainder of the battalion Vincedores and their families, including women and children (176 persons), were inoculated with a vaccine killed with 0.5 per cent phenol.[3]   At this period the culture material on hand

had been nearly exhausted; a culture much inferior to the first was utilized, and the number of vaccinations to be carried out necessitated diluting the small amount of material available; hence the resulting vaccine preparation (Vaccine B) was only one-fifth the strength of that previously used in Quito (Vaccine A).

Of the 149 soldiers who were vaccinated in Quito, three who had received 1 c.c. of vaccine (Vaccine A) later contracted yellow fever in Guayaquil 35, 93 and 99 days, respectively, after vaccination; the first case was fatal (Table 3).

TABLE 3.

Yellow Fever Incidence among Vaccinated Persons.

Name	Age Years	Vaccine Preparation Employed	Amount Injected C c	Date of Vaccination	Date of Onset of Yellow Fever	Time Between Vaccination and Onset, Days	Result
M. M.	34	Vaccine A	1	9/24/18	10/29/18	35	Died 7th day
M. B.	22	Vaccine A	1	9/24/18	10/29/18	93	Recovered
E. M.	28	Vaccine A	1	9/24/18	1/ 4/19	99	Recovered
B. R.	40	Vaccine B	1	10/ 8/18	10/14/18	6*	Died 5th day
I. M. C.	10	Vaccine B	1	10/ 8/18	10/28/18	20	Died 9th day
M. P. V.	19	Vaccine B	1	10/ 8/18	11/ 6/18	29	Recovered

*An instance in which the patient had contracted yellow fever before the vaccination had conferred any immunity.

Of the 176 soldiers and their families vaccinated after their arrival in Guayaquil, one, who had received 1 c.c. of Vaccine B, developed yellow fever six days after vaccination and died on the fifth day of illness. This case may be regarded as a control, since the vaccination could not have conferred any protection within this short period. Two more cases, occurring among the persons vaccinated in Guayaquil with Vaccine B, one twenty days (fatal) and the other twenty-nine days (recovered) after the vaccination, demonstrate either that the amount of vaccine given was insufficient, or that these individuals were unusually susceptible to the leptospira infection—a phenomenon observed also among vaccinated animals.

It may be noted that among the persons receiving 2 c.c. of Vaccine A, none contracted yellow fever, and that of the three persons who developed yellow fever notwithstanding the injection of 1 c.c. of Vaccine A, two recovered.   The incidence of yellow fever among the vaccinated and that among the unvaccinated nonimmune population of Guayaquil may be roughly compared by referring to the yellow fever statistics published by the Department of Health of Guayaquil for 1918–1919[3] (Table 4).   During the months of October, November and December, 1918, and January, February and March, 1919, 386 cases of yellow fever occurred in Guayaquil, with 217 deaths (56 per cent.).   The nonimmune population of the city, which is not accurately known, must have been comparatively small; hence the yellow

TABLE 4.

Yellow Fever Incidence at Guayaquil in 1918–1919.

Year	Month	Number of Cases Occurring	Deaths	
			Number	Percentage
1918	October	72	41	57
	November..........	81	43	52
	December.................	88	46	52
1919	January..........	85	51	60
	February.	43	22	52
	March	17	14	80

fever incidence among the vaccinated (five cases among a total of 427) was strikingly low in comparison with that among the unvaccinated nonimmune population, and the mortality was also low (33 per cent. of the Vaccine A group, and 40 per cent. among both groups).

Following the inauguration of a rigorous antistegomyia campaign (December, 1918) throughout Ecuador under the department of health (Dr. Becerra) with the cooperation of the International Health Board (Dr. M. E. Connor), the yellow fever incidence became gradually so reduced that in May, 1919, only a single case occurred, and

3. Subsequently 102 additional vaccinations were carried out (with more concentrated vaccine), making the total number of vaccinations in Ecuador 427. See the Boletin Trimestral de la Oficina de Censo, Estadistica, y Despacho Municipal de Guayaquil 1, Nos. 1 and 2, 1919.

since that date the country has remained free from the disease;[4] hence the prophylactic value of the vaccination could no longer be tested in Ecuador.

During 1919–1920, the northern districts of Peru, the countries of Central America, and certain localities in Mexico have been invaded by yellow fever, and the vaccination has been practiced on a large scale by several sanitary experts (5,000 vaccinations in Salvador and Guatemala by Dr. T. C. Lyster, Dr. C. A. Bailey, and Dr. E. I. Vaughn in 1920 of the International Health Board), who will no doubt publish their findings in time.

The concentration of the vaccine now prepared at the Rockefeller Institute is such that 1 c.c. contains about 2,000,000,000 organisms. About 0.2 per cent. agar is being used in the culture medium, and the culture is diluted with twice or more its volume of saline solution at the time of making the vaccine. The presence of agar in the vaccine is objectionable, as it may rarely remain unabsorbed, leading to sterile abscess formation; notwithstanding repeated efforts, however, we have not yet succeeded in growing *Leptospira icteroides* abundantly in a liquid medium; but by careful massage at the site of inoculation, the absorption of the agar is facilitated and the abscess formation prevented. Dr. Lyster and his associates, in their series of 5,000 vaccinations, have observed only six instances of sterile abscess, which occurred two weeks after inoculation.

SUMMARY.

From the results of vaccination in guinea-pigs, it may be concluded that when sufficient quantities of the killed culture are given, these animals are usually rendered resistant to a subsequent infection with leptospira. The degree of protection, however, is not strictly proportional to the amounts of the vaccine inoculated.

As regards the vaccination of human beings, thus far the results are distinctly encouraging; but we realize that many more observations will be needed before a final decision of its value can be arrived at.

4. Comercio Internacional, Guayaquil. January, 1920, page 53. Connor, M. E.: Yellow Fever Control in Ecuador, J. A. M. A. **74**: 650 (March 6) 1920; **75**: 1184 (Oct. 30) 1920.

EXPERIMENTAL PRODUCTION OF GIGANTISM BY FEEDING THE ANTERIOR LOBE OF THE HYPOPHYSIS.

By EDUARD UHLENHUTH.

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Perhaps the most reliable information as to what may be the function of the hypophysis (or any other endocrine gland) may be expected to be obtained through experiments on the extirpation and transplantation of the gland. The majority of these experiments have shown in a rather conclusive way, that growth and development are inhibited in the partial (mammalians) or total (amphibians) absence of the anterior lobe; an increase of the rate of growth ensues if anterior lobes are grafted to the animals.[1] Particularly clear are the results obtained in amphibians. As shown by Smith[2] and by Allen,[3] the extirpation of the anterior lobe of the hypophysis results in an inhibition of growth and metamorphosis of the operated tadpoles. Recently Allen[4] has shown that grafting the anterior lobe of adult frogs on tadpoles causes an acceleration of growth and development in normal larvæ, and that it also restores the power of growth and development after they had been lost through extirpation of the anterior lobe.

These experiments seem to demonstrate that the anterior lobe of the hypophysis is the organ which makes growth possible during the normal growth period of life. They do not afford, however, any evidence as to whether the substance of the anterior lobe can cause growth to continue beyond the period of life in which, under normal conditions, the ability of growth is lost and whether in this way the anterior

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[1] For a more complete discussion of the literature see Uhlenhuth, E., The rôle of the internal secretions in growth and development, in a book on Internal secretion and metabolism, edited by L. F. Barker and R. G. Hoskins (in press).

[2] Smith, P. E., *Science*, 1916, xlix, 280; *Anat. Rec.*, 1916–17, xi, 57.

[3] Allen, B. M., *Science*, 1916, xliv, 755.

[4] Allen, B. M., *Science*, 1920, lii, 275.

lobe substance can increase the size of the individual over the normal "maximum" size of the species. It is well known that growth of every individual stops as soon as the specific size of the species is reached. Many problems pertaining to this phenomenon would appear in a new light if it were possible to cause gigantism by a particular substance.

Clinical observations point to the conclusion that at least one form of gigantism is caused by an excessive production of anterior lobe substance; nevertheless, attempts to produce experimental gigantism have so far been unsuccessful. The only way to attack this problem seems to be the feeding of the anterior lobe substance by mouth. Such experiments have been attempted in large numbers but the results have for the most part been contradictory and difficult to interpret. The majority of investigators have merely desired to determine whether or not feeding of anterior lobe modifies in a specific way the rate of growth. It will be pointed out later that the greatest care is necessary in the interpretation of results obtained from feeding experiments. From the more recent feeding experiments, and especially those performed by Hoskins and Hoskins[5] and by Smith[6] on tadpoles, by Robertson[7] and his coworkers on white mice, and by Wulzen[8, 9] on chickens, most students of endocrinology have concluded that the anterior lobe substance retards growth in early periods of life, while later on it may cause an acceleration of growth.

But in these experiments none of the animals fed with anterior lobe developed into giants, except in two cases in which the slightly greater size of the experimental animals may have been due to the effect of the anterior lobe substance. Robertson and Ray[10] claim that they obtained unusually large mice, when the feeding of anterior lobe substance was started at an age of 4 weeks and discontinued at an age

[5] Hoskins, E. R., and Hoskins, M. M., *Endocrinology*, 1920, iv, 1.

[6] Smith, P. E., *Univ. California Pub., Physiol.*, 1918, v, 11.

[7] Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 385, 397, 409. Robertson, T. B., and Delprat, M., 1917, xxxi, 567. Robertson, T. B., and Ray, L. A., 1919, xxxvii, 393, 427, 455.

[8] Wulzen, R., *Am. J. Physiol.*, 1914, xxxiv, 127.

[9] Wulzen, R., *J. Biol. Chem.*, 1916, xxv, 625.

[10] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1919, xxxvii, 455.

of 12 weeks. In experiments in which the chicks were fed anterior lobe from an early stage, Wulzen[9] succeeded in raising one anterior lobe-fed cock which weighed 1,882 gm. as against the weight of only 1,597 gm. of the control animal; no normal cock raised by Wulzen grew to the size of the hypophysis-fed bird. Wulzen, however, raised only one cock of this kind; moreover, no records are given in the paper as to the normal maximum size of that race of chickens, and, therefore, it is not certain that this cock could be actually considered a true giant.

From the experiments to be reported in this paper, it will become evident that at least in one group of cold blooded animals, namely in salamanders, feeding of anterior lobe leads to the attainment of a size considerably in excess of not only the normal "average" size, but of even the greatest known size of the two species (*Ambystoma opacum* and *Ambystoma tigrinum*) employed in these experiments.

Critical Remarks Concerning the Methods of Feeding.

It is usually stated that in order to obtain reliable results in feeding experiments of this kind, only small amounts of the glandular product should be added to an otherwise normal diet. I, however, am of the opinion that this method, although considered at present as the standard method, cannot, at least under all conditions, give conclusive results. It is possible that a gland may contain a specific growth-promoting principle and yet may not give, with the above mentioned method, positive results if the amount of the hormone fed is too small. We do not know how much of a supposedly hormonic substance is contained in a definite amount of the fresh product, nor do we know how much of it may be required to produce gigantism or accelerated growth. While most hormones act in small quantities, it would be premature to conclude that all hormones should act in small amounts. The main point in experiments of this kind is to be able to prove that the control animals are fed in such a way as to show the maximal rate of normal growth known to exist in the species experimented upon.

In my experiments the controls were fed exclusively the normal diet, while the experimental animals were fed exclusively on the anterior lobes of the hypophysis of cattle. This method is based on the

following considerations. If earthworms are a complete diet for salamanders, the worm-fed animals should grow at the maximum rate characteristic of the species, provided that the calories and vitamines are in sufficient quantity to make the maximum rate of growth at that particular temperature possible. If by chance the anterior lobe should have a higher food value than the earthworm, this could not result in a better growth as long as the food value of the earthworm suffices for the maximum normal growth. If the anterior lobe of the hypophysis contains only ordinary food substances and no specific hormone for growth, the rate of growth of the animal should not exceed the specific maximum rate.

Hence only two conditions had to be provided for. First, all animals, controls and experimental, had to be kept at the same temperature, a condition which was carefully provided for. Second, all animals were given as much food as they desired to take. This condition was approached as nearly as possible.

The worm-fed animals took the worms readily and voluntarily. The gland-fed animals, however, had to be fed by pushing the pieces of gland into their mouths; yet it was possible to adjust the quantity of food according to the appetite of the animals. They yield readily if·the food is gently pressed against their jaws, and swallow the food quickly when they are hungry; while they push the food back by means of the tongue or eject it when it is forced into the mouth, if they are not hungry.

The quantity of food taken up by the controls was generally greater than the quantity of hypophysis taken by the experimental animals.

Characteristics of Growth of Normal Salamanders.

Two species of salamanders were employed in these experiments, *Ambystoma opacum* and *Ambystoma tigrinum*. Since little is known about the normal growth of these animals, some data pertaining to it may be recorded here.

Large numbers of *Ambystoma opacum* were reared from eggs in my laboratory and the growth of the species has been recorded over long periods. The longest record I possess is that of four animals which at present are 3 years (161 weeks) of age and were raised from eggs of

two different females. Many animals of this species were observed for a period of over 2 years and behaved essentially the same way. Fig. 1 shows the growth curves of the four 3 year old animals and illustrates the most important characteristics of the growth of that species. Although each animal, during the larval period, was treated in a different way (D_1 normal, E_3 underfed, $W_{Ca}5$ kept in water + a small amount of Ca lactate, $W_{Na}4$ kept in water + a small amount of Na lactate) all four curves are about the same. The most rapid growth takes place during the larval period (first period of growth); at the end of this period a decrease in size is noticeable which may last for several weeks and corresponds to metamorphosis. The second period of growth lasts from after metamorphosis till the first breeding season, at which the animals are about 1 year old. The beginning of the breeding season in the male may be recognized by the swelling and reddening of the cloacal region and in both males and females by the cessation of food intake. During the second period of growth the animals continue to grow at a fairly high rate; during the first as well as the following breeding seasons little growth or even a decrease in size may be noticed. The third period of growth begins after the first breeding season and lasts till the third breeding season, and is characterized by slow growth. No records are available to determine the behavior after the third breeding season, but to conclude from the normal maximum size of the species growth seems to be nearly completed at the end of the third period. The control animals of the hypophysis-fed series exhibit a similar type of growth (Fig. 2), although they were from a different season (1918). It is safe to assume that this is the type of growth characteristic for the species *Ambystoma opacum*. The most important feature of it, in connection with the following experiments, is the considerable slowing down of growth after the first breeding season.

As to the normal size of the species, the following records are available. The largest animal on record in my laboratory was 115 mm. long at an age of 79 weeks (it was one of the controls of the hypophysis-fed animals). The largest animal among fifteen specimens of from 2 to 3 years of age, still alive at present, measures 113.5 mm., the average size of these fifteen animals being 103.5 mm. (the small size of some of them may be due to the abnormal conditions under
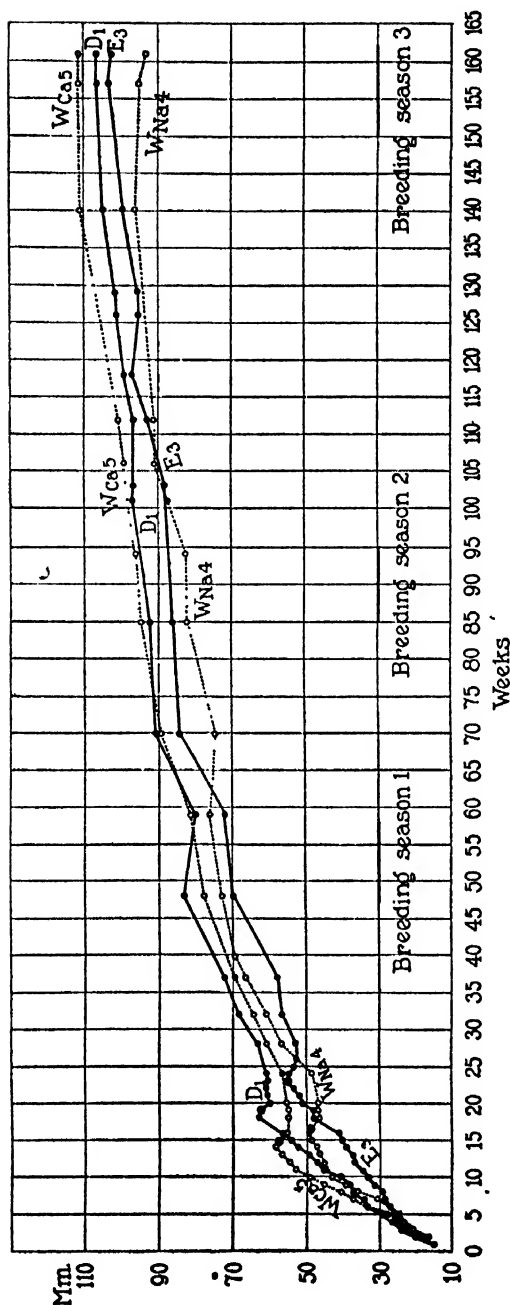
Fig. 1. Growth of four specimens of *Ambystoma opacum* during a period of 3 years. All four animals were fed normal diet, but E_3 was underfed during the larval period and W_{Ca} 5 and W_{Na} 4 were kept in water + small amounts of Ca lactate and Na lactate respectively during the larval period. The figure is drawn to a smaller scale than Figs. 2, 3, 4, and 5.

which they were kept during the larval period). Two breeding females, collected recently outdoors, measured 112 mm. and 106 mm. respectively.[11] Cope,[12] in his book on North American batrachians, mentions the specimen, from which he described the species, as measuring 3.8 inches (about 100 mm.). The largest animal found in the collection of the American Museum of Natural History[13] measured 117.7 mm.; the average of the eight largest animals was 100.3 mm.

FIG. 2. Experiment 1 (*Ambystoma opacum*, 1918, Series XIV). The unbroken line indicates the growth of the controls, the dotted line that of the anterior lobe-fed animals.

To conclude from these data the maximum size of the species is nearly 118 mm., while specimens measuring more than 115 mm. are of very rare occurrence.

[11] I am obliged to Mr. George P. Engelhardt, of the Brooklyn Museum, for this record.

[12] Cope, E. D., The batrachia of North America, Washington, 1889.

[13] I wish to express my appreciation to Miss M. Dickerson for giving me the opportunity of examining the collection of salamanders at the American Museum of Natural History.

I have no other records concerning the growth of the species *Amby-stoma tigrinum* except those afforded by the control animals of these experiments. These give information on the growth during a period of only $1\frac{1}{2}$ years (84 weeks), the present age of the animals of Series A, XLVI, and LV. Apparently this species, as illustrated in Figs. 3, 4, and 5, behaves very much in the same way as the species *Ambystoma opacum*, growth being most rapid during the larval period, and continuing at a fairly high rate till about the end of the 1st year, after which it becomes rather slow.

It is not so simple to determine the maximum size of this species as in the case of *Ambystoma opacum*, since there are two races of the tiger salamander, a western and an eastern one, which are very different in respect to the type of growth. The western race seems to be naturally a giant race; this is true at least for the specimens which metamorphose from the well known neotenous larvæ found in the western lakes. This condition, however, is doubtlessly caused by disturbances of the endocrine system and is rather pathological than normal. My experiments, performed with eastern animals, cannot be checked by means of these giants although they are apt to throw some light on the gigantism of the neotenous specimens and those that have metamorphosed from neotenous larvæ. Cope in describing a large number of specimens of *Ambystoma tigrinum* mentions that the largest specimen among them measured 10 inches (about 244 mm.) and that De Kay described a still larger one measuring 11 inches (about 280 mm.). The largest specimen among 55 neotenous larvæ which I myself collected in the vicinity of Tolland, in the Colorado Rocky Mountains, measured 257 mm., while all the metamorphosed specimens collected in the same locality were much smaller. As pointed out above, I have used in my experiments only eggs that were from females of the eastern race. Unfortunately, I have not reared enough animals of this species to form a conclusive idea as to the normal maximum size of the species. But the largest individual on record in my laboratory (one of the controls of these experiments) measures 200 mm. at an age of 84 months; it is, however, still growing, although very slowly. In the collection of the American Museum of Natural History I found the largest specimen among the nine largest animals, eastern as well as western, to measure 208.7 mm.
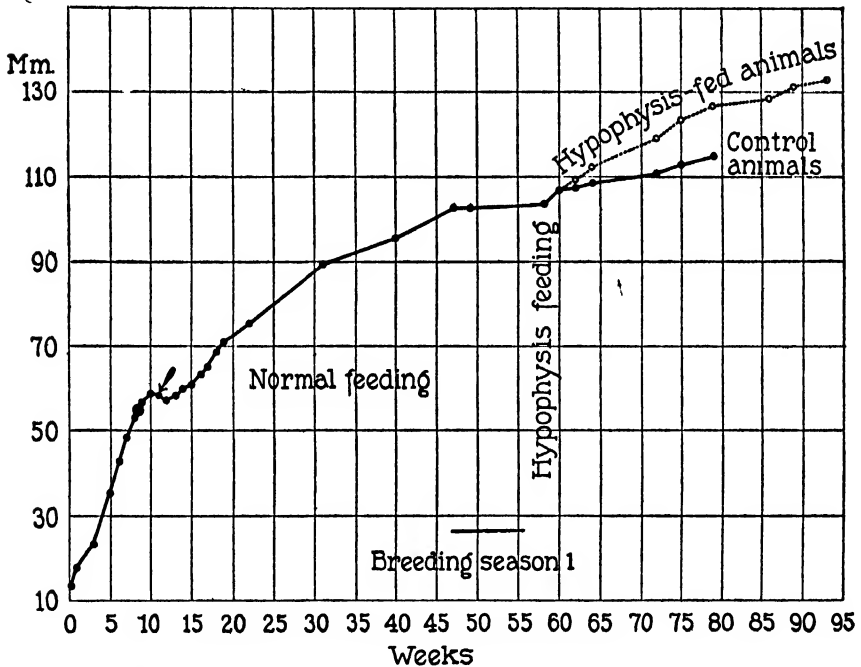
FIG. 3. Experiment 2 (*Ambystoma tigrinum*, 1919, Series XLVI). The unbroken line illustrates the growth of the controls, the dotted line that of the anterior lobe-fed animals.

FIG. 4. Experiment 3 (*Ambystoma tigrinum*, 1919, Series LV). The unbroken line represents the growth of the controls, the dotted line that of the anterior lobe-fed animals.
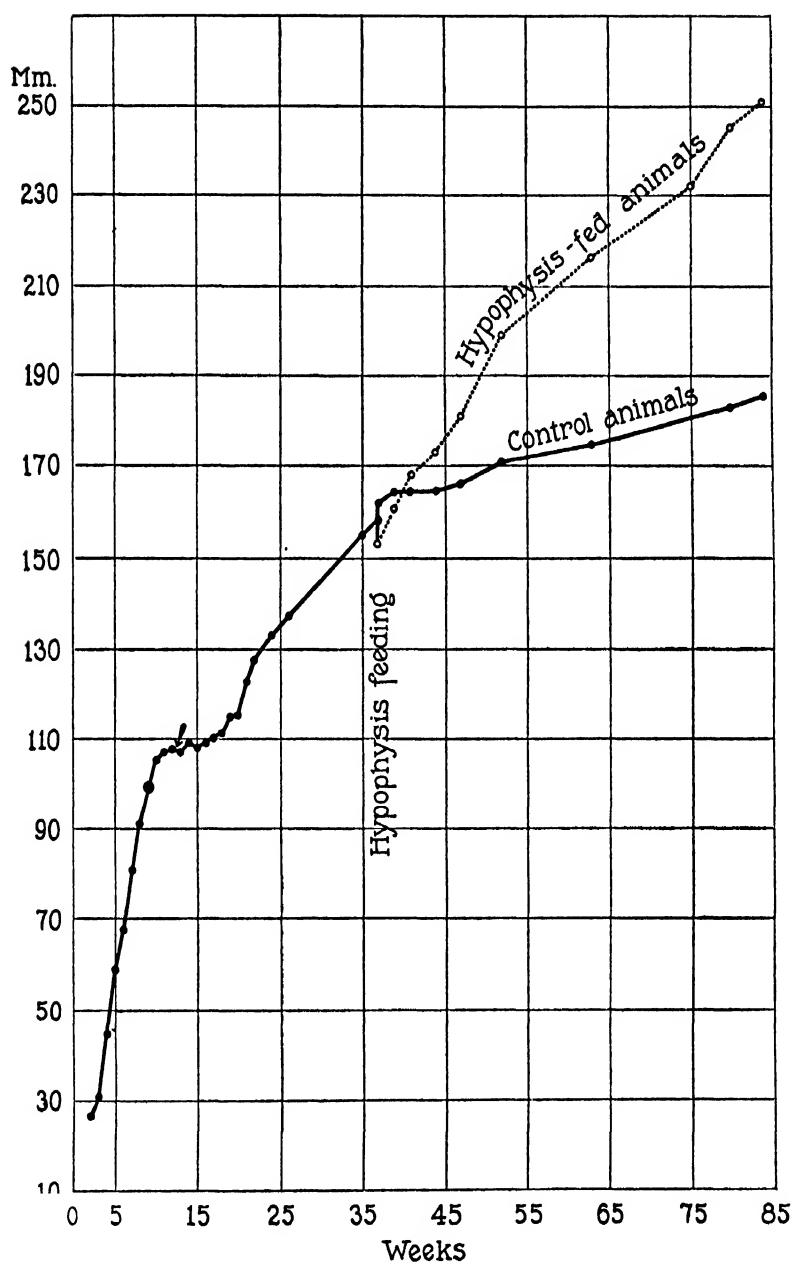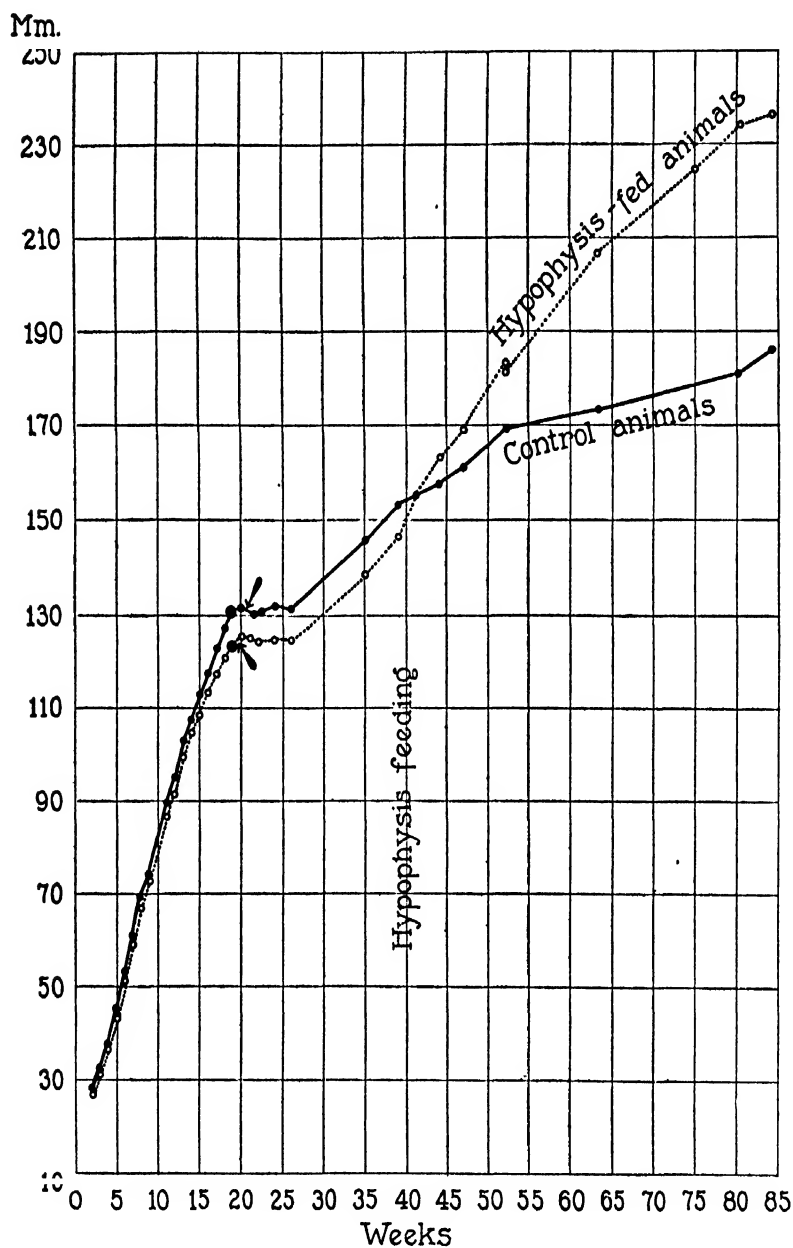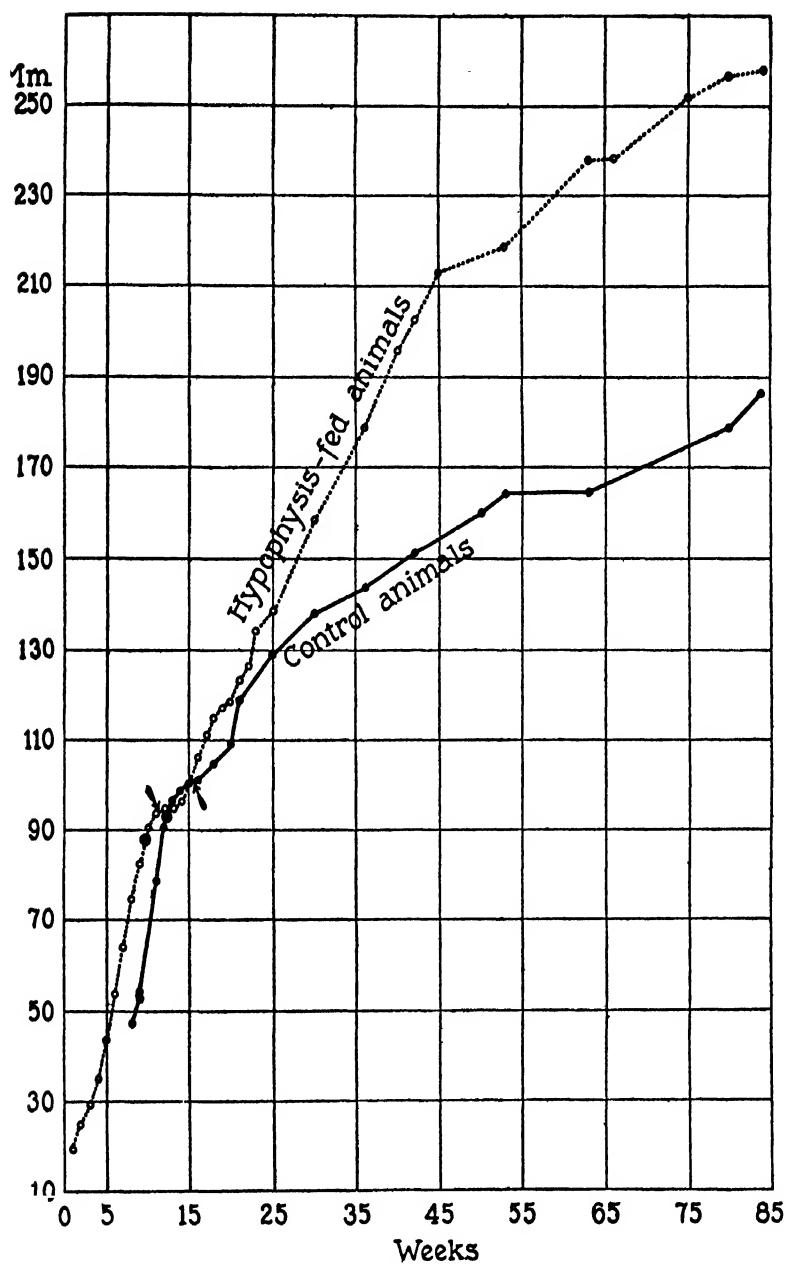
FIG. 5. Experiment 4 (*Ambystoma tigrinum*, 1919, Series A and XXXVII). The unbroken line represents the growth of the controls, the dotted line that of the anterior lobe-fed animals.

while the average was 200.8 mm. From these data it seems that while the largest specimen that could be found among eastern animals of this species measured 208.7 mm., pathological conditions of the endocrine system may produce giants of a maximum size of 280 mm.

The Growth of the Hypophysis-Fed Animals.

Experiment 1.—Of four normal metamorphosed specimens (Series XIV) of *Ambystoma opacum* which had been reared in the laboratory from eggs of the same female (Brood 1918) two were started on a diet of anterior lobe of cattle hypophysis at an age of 60 weeks, *i.e.* at the beginning of the third period of growth; the other two animals were controls and were kept on an abundant diet of earthworms. The size of the animals at the beginning of the experiment may be seen from Table I.

TABLE I.

Hypophysis-Fed Ambystoma opacum, Experiment 1.

	Animal No.	Size of animals.		
		60 weeks.	72 weeks.	End of experiment.
		mm.	*mm.*	*mm.*
Control.......................	4	102.1	108.1	108.1 at 72 weeks.
•	1	111.0	113.2	115.0 " 79 "
Anterior lobe-fed animals..........	2	110.4	120.6	130.5 " 105 "
	3	103.1	118.8	138.0 " 101 "

The rate of growth of the anterior lobe-fed animals, after 2 weeks of feeding, rose over that of the controls and continued at such a height as is usually observed only in the second period of growth (Fig. 2). The two control animals, although they were fed on an abundant diet, continued to grow at a slow rate (Fig. 2) characteristic of the third period of growth (Fig. 1). In consequence of their rapid growth the anterior lobe-fed animals, 12 weeks after the beginning of the experiment, had reached a size exceeding that of the largest animals of this species on record (Table I, 72 weeks). At the end of the experiments (caused by the death of the animals) the largest anterior lobe-fed animal measured 138 mm., the smaller one 130 mm., while the controls had reached a size of only 115 and 108 mm. respectively. Unfortu-

nately both of the controls were lost before the anterior lobe-fed animals had reached such an extraordinary size, but from our experiences with many other animals as well as from the maximum size of this species it is safe to conclude that the growth of the controls in this experiment was nearly completed. On the other hand, the size of the anterior lobe-fed animals exceeded the normal maximum size of the species so much as to leave no doubt that both of them were giants. The size of the larger experimental giant exceeded the size of the largest normal animal of this species on record by 20 mm., and the size of the smaller experimental giant exceeded the largest normal animal on record by 12 mm. It is certain that both animals would have become still larger had they survived, since they were still growing before they died.

TABLE II.

Hypophysis-Fed Ambystoma tigrinum, Experiment 2.

	Animal No.	37 weeks.	63 weeks.		84 weeks.	86 weeks.
		Size.	Size.	Weight.	Size.	Weight.
		mm.	*mm.*	*mg.*	*mm.*	*mg.*
Controls..................	2	152.4	163.0	22,400	171.5	34,800
	3	163.5	183 0	31,200	190.0	40,000
	4	167.9	179.0	27,900	197.0	35,200
Anterior lobe-fed animals.....	5	163.5	233.0	57,200	268.0	72,300
	6	142.5	199.0	50,500	233.0	78,000

Similar results were obtained when metamorphosed specimens of the species *Ambystoma tigrinum* were fed on anterior lobe.

Experiment 2.—Five larvæ (Series XLVI) were reared from eggs of the same female and fed on earthworms. At the end of the second growth period, about 24 weeks after metamorphosis, when the animals were 37 weeks old, three of them, among them the largest one of the series, were kept on earthworms, while two, among them the smallest one, were started on an exclusive diet of anterior lobe (for initial sizes see Table II).

Although the control animals were fed on an abundant supply of earthworms, their rate of growth remained low as compared with that during the second period of growth. The animals fed on anterior

lobe, however, commenced to grow so rapidly that their rate of growth soon exceeded that which prevailed even during the earlier period of life (see Fig. 3). At an age of 63 weeks, 26 weeks after the beginning of the experiment, the smallest animal fed on anterior lobe was 20 mm. larger than the largest control animal, although at the beginning of the experiment it was 10 mm. smaller than the smallest control specimen; its weight was more than twice the weight of that of the smallest control animal. At an age of 84 weeks the largest animal of this series fed on anterior lobe measured 268 mm. as compared with 197 mm., the latter being the size of the largest control animal. Hence the size of the largest anterior lobe-fed animal exceeds at present the size of the largest normal animal, on record in the laboratory, by 68 mm. and the size of the largest specimen of the eastern race of *Ambystoma tigrinum* by 60 mm. It is 12 mm. smaller than the largest specimen of the western race on record, which, however, cannot be considered normal, as pointed out above. Both the controls and the anterior lobe-fed animals are still growing and at the present time the rate of growth of the anterior lobe-fed specimens is still higher than that of the control animals (see Fig. 3). It is possible, therefore, that in a short time, the largest anterior lobe-fed animal will exceed in size even the largest western specimen known to the writer.

Experiment 3.—Six larvæ (Series LV), of the same brood from which the animals of Experiment 2 were taken, were raised on earthworms. At the end of the second period of growth, about 19 weeks after metamorphosis and at an age of 39 weeks, three of them (Nos. 1, 2, and 4), among them the largest one, were used as worm-fed controls; the other three animals, among them the smallest one, were kept on a pure anterior lobe diet (Table III). The largest of these latter animals died soon after commencement of the experiment.

The results were similar to those obtained in Experiment 2 and may be seen from Table III and Fig. 4. At an age of 84 weeks the larger one of the two hypophysis-fed animals measured 51 mm. more than the largest control animal, and 44 mm. more than the largest animal of the eastern race of the species known to the writer. The hypophysis-fed specimens as well as the control animals are still growing, but at present the growth of the anterior lobe-fed specimens of this series is less vigorous than that of the controls.

Experiment 4.—In this experiment (Series XXXVII₂ and Series A) the controls were underfed and therefore cannot be used for comparison. Both the experimental as well as the control series were obtained from the eggs of the same female; but the control animals were underfed for several weeks during the larval period and the experimental animal, which was fed abundantly on anterior lobe from the beginning of its life, was kept in distilled water during the larval period, which tends to retard growth. The results are seen in Fig. 5. The anterior lobe-fed animal measured 258 mm. at an age of 84 weeks; *i.e.*, 58 mm. more than the largest normal animal kept in the laboratory and 50 mm. more than the largest specimen of the eastern race. At present, however, it is growing very slowly and less vigorously than the animals of Series A.[14]

TABLE III.

Hypophysis-Fed Ambystoma tigrinum, Experiment 3.

	Animal No.	39 weeks.	63 weeks.		84 weeks.	86 weeks.
		Size.	Size.	Weight.	Size.	Weight.
		mm.	*mm.*	*mg.*	*mm.*	*mg.*
Controls..................	1	163.8	182.2	27,400	200.2	38,500
	2	156.1	176.4	30,000	182.0	40,200
	4	137.2	161.5	22,500	176.5	31,100
Anterior lobe-fed animals ...	3	136.4	208.5	54,200	251.6	95,700
	5	146.7	204.0	60,000	221.5	84,300
	6	156.1	Dead.			

[14] Since this paper went to press, Dr. Leonhard Stejneger, head curator of the United States National Museum, was kind enough to go over the entire collection of the museum and let me have the measurements of the largest specimens of the species *Ambystoma opacum* and *Ambystoma tigrinum*. Since the measurements are based on a far larger collection of material than my own data reported in the preceding pages, they are of greater importance and will be added below. They confirm the opinion expressed in this article that feeding the anterior lobes of hypophysis produces gigantism in salamanders.

Ambystoma opacum.	
Normal.	Hypophysis-fed.
mm.	*mm.*
119	138
116	130

DISCUSSION.

The feeding of anterior lobe to metamorphosed salamanders has two different effects on the growth of the animals: first, it increases the rate of growth over that of normal animals; and, second, it maintains growth after the normal "maximum" size of the species has been attained. Have we any reason to consider these effects as the result of a "specific" growth-promoting hormone?

As to the first point, we cannot be quite certain that the rate of growth of our control salamanders was the highest rate of growth which can be obtained with a normal diet. Unfortunately the data available on the growth of metamorphosed salamanders are not numerous enough to decide if earthworms form a sufficiently complete diet for the two species in question. Although it is quite certain that these animals do not require a plant diet, not enough food materials have been tested by the writer to state on which food they nat-

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The two largest hypophysis-fed specimens of *Ambystoma opacum* exceed in size the largest known animal of this species by 19 and 11 mm. respectively.

Ambystoma tigrinum.	
Eastern race.	
Normal.	Hypophysis-fed. (Age 88 weeks.)
mm.	*mm.*
235	273.5 (Experiment 2, No. 5)
223	235.5 (" 2, " 6)
	257.1 (" 3, " 3)
	226.2 (" 3, " 5)
	263.0 (" 4, " 2)

Among the five hypophysis-fed specimens of *Ambystoma tigrinum* three are considerably larger than the largest known specimens of the eastern race, the largest hypophysis-fed specimen exceeding in size the largest eastern specimen by 28.5 mm.

There is, as stated above, a western giant race of *Ambystoma tigrinum*. Dr. Stejneger gives 258, 265, 285, 285, and 292 mm. as measurements of the five largest specimens of this race. The largest of our hypophysis-fed animals, which has grown 5.5 mm. in the last 4 weeks and measures at the time of writing 273.5 mm., has already outgrown two of the western giants and will soon outgrow the three others if it continues to grow at the present rate.

urally grow best.   Feeding of calf thymus or posterior lobe of hypophy-
sis of cattle does not induce any growth equal to that of worm-fed
animals.   In the feeding of salamander larvæ I have used many differ-
ent substances (frog muscle, beef muscle, lymph gland, parathyroids,
thymus, spleen, cheese, milk) with and without the addition of normal
food; it is certain that in larvæ no other diet can produce a rate of
growth higher than that produced by earthworms.   But, of course,
the metabolic processes involved in growth may be quite different
for the larvæ and the metamorphosed animals.

On the other hand, if we look at the curves of the various series, it
is noticeable that the curves for the worm-fed animals are very much
like the normal growth curves of most other animals whose growth
has been studied carefully.   In particular they show the gradual flat-
tening out of the growth curves of warm blooded animals.   The
curves of the anterior lobe-fed animals, especially those of *Ambystoma
opacum* (Fig. 2), with their sudden rise above the flat level of the
normal curve, differ from this general normal type.

Although at present we must postpone more definite conclusions,
it seems at least probable that the rate of growth of the animals fed
exclusively on anterior lobe is the result of a specific growth-promoting
hormone contained in the anterior lobe of the hypophysis.

It is beyond doubt that the size of the hypophysis-fed animals
exceeds the "maximum" size of the species.   The animals fed on ante-
rior lobe are true giants.   The hormone of the anterior lobe is not only
able to accelerate growth, but also—and this is of far greater impor-
tance—possesses the property of maintaining growth when the nor-
mal size of the species has been reached.   The production of experi-
mental gigantism by means of feeding anterior lobe proves that this
organ contains actually a specific substance which can overcome the
obstacles which are responsible for the discontinuation of growth
when the normal size is reached.

These experiments do not give any clue as to whether the cells of
the body are directly affected by the hormone or whether this hormone
acts by the intermediation of another organ.   In view of the results
obtained with single cells. it is probable that the cells are not directly
affected by the anterior lobe hormone.   It was found by Shumway, by

Chambers, and by Nowikoff[15] that the division rate of Protozoa is not increased if anterior lobe extract is added to the culture medium. The same is true for the cells of warm blooded animals. In as yet unpublished experiments Carrel found that in tissue cultures the growth of the cells of warm blooded animals cannot be accelerated if anterior lobe extract is added to the culture medium.[16] Our experiments show, however, that the cells of the organism are still capable of dividing at a time at which the organism as a whole has stopped to grow. Apparently the cessation of growth of the organism as a whole is not caused by a fundamental property of the cell to become incapable of growth, after growth has been going on for some time. This corroborates the well known tissue culture experiments of Carrel which would suggest that the cell protoplasm, if kept under proper conditions, can go on dividing for indefinite periods. Since the hypophysis-fed salamanders are still growing, it is impossible, at present, to say how long the anterior lobe hormone can maintain, within the organism as a whole, a condition permitting active growth of the cell. But it is possible to say that the size of the individual is not an obstacle to the further growth of an organism. It has frequently been claimed that the size of every species is determined by the mechanics and statics of the substances constituting the body of the organism. Although these principles may be among the reasons why the organism stops growing after it has attained a definite size, they cannot be the only reason; the occurrence of giants shows that at least a considerable increase in the specific size of the species would result if the size of the organism would be determined by the mechanic and static principles alone.

As pointed out above, the evidence that anterior lobe feeding can produce gigantism in warm blooded animals is not yet sufficient. It would be of great importance if it should be demonstrated that this difference is due to the difference in the body temperature of amphibians and warm blooded animals. Lenz[17] has recently called atten-

[15] Shumway, W., *J. Exp. Zool.*, 1917, xxii, 529. Chambers, M. H., *Biol. Bull.*, 1919, xxxvi, 82. Nowikoff, M., *Arch. Protistenk.*, 1908, xi, 309.

[16] I am indebted to Dr. Alexis Carrel for permission to quote these, as yet unpublished, experiments, which were carried out in Dr. Carrel's laboratory.

[17] Lenz, F., *Münch. Med. Woch.*, 1919, lxvi, 992.

tion to the fact that in acromegaly the tendency to abnormal growth is confined to the prominent parts of the body, in which the temperature is lower than in the main body.

SUMMARY.

1. Metamorphosed salamanders of the species *Ambystoma opacum* and *Ambystoma tigrinum* were fed on a pure diet of the anterior lobe of the hypophysis of cattle; the controls were fed on an abundant diet of earthworms.

2. The rate of growth of the animals fed on the anterior lobe of the hypophysis was greatly increased over the rate of growth of normal animals.

3. Growth of the animals fed on anterior lobe did not cease after they had reached the normal "maximum" size of the species, and experimental giants were produced.

4. The largest animal of the species *Ambystoma opacum* fed on anterior lobe of the hypophysis was 19 mm. larger than the largest normal animal of this species known to the writer; the largest animal of the species *Ambystoma tigrinum* fed on anterior lobe is at present about 28 mm. larger than the largest normal animal of the eastern race of this species known to the writer.

INDUCED ATROPHY OF HYPERTROPHIED TONSILS BY ROENTGEN RAY.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Early in the study of the biologic effects of the roentgen ray it was noted that the lymphoid tissue was easily affected by this agent.[1] After long exposures of animals, both the lymphoid tissue and the circulating lymphocytes were found to be almost completely destroyed. This observation led to the use of the roentgen ray as a therapeutic agent in the treatment of lymphatic leukemia and in reducing enlarged spleens and glands occurring in other diseases. In this laboratory it was shown experimentally that the susceptibility of the lymphoid tissue was so much greater than the susceptibility of other tissues that by the judicious use of this agent the major portion of the lymphoid tissue of the body could be destroyed without appreciable damage to other tissues.[2]

The recent emphasis laid on the tonsil as a seat of focal infections has suggested that these lymphoid organs might be reduced by the roentgen ray and their shrinkage be accompanied by proper drainage of the crypts with resultant clearing up of the infections.

TECHNIC OF TREATMENT.

The factors governing the dose of roentgen ray given to the region of each tonsil were as follows: The spark gap, measured between points, was 8 inches; 5 milliamperes; 10 inches distance from the target to the highest point of skin exposed. The time of exposure varied from three to seven minutes, according to the age of the patient. The

1. Heineke, H.: Mitt. a. d. Grenzgeb. d. Med. u. Chir. 14: 21, 1905.

2. Murphy, J. B.: Heteroplastic Tissue Grafting Effected Through Roentgen-Ray Lymphoid Destruction, J. A. M. A. 62: 1459 (May 9) 1914. Murphy, J. B., and Ellis, A. W. M.: J. Exper. Med. 20: 397, 1914. Taylor, H. D., Witherbee, W. D., and Murphy, J. B.: J. Exper. Med. 29: 53 (Jan.) 1919.

ray was filtered through 3 mm. of aluminum. The approximate value of this dose was from 1 to $1\frac{3}{4}$ skin units. The patient to be treated was placed on a table in such a position that the ray entered under the angle of the jaw and penetrated through the soft tissues to the region of the tonsil. The area exposed on each side was about 3 square inches, the surrounding parts being protected by heavy sheet-lead. For young children a special board was used with retaining straps, and the head secured by means of a gauze bandage.

<div align="center">RESULTS.</div>

Material.—This report is based on the result of the foregoing treatment on forty-six patients ranging from $3\frac{1}{2}$ to 45 years of age, all showing more or less hypertrophy and disease of the tonsils and surrounding tissues. No patients were treated during a time when the throat showed evidence of acute infection. The state of the tonsils varied from those with deep crypts, ragged surface, purulent, mucopurulent or cheesy exudates, to a condition of marked hypertrophy with other pathologic states less marked.

Tonsils.—In all but four cases the treatment was followed by marked improvement. In the majority of cases, two weeks after the exposure to the roentgen ray a distinct shrinkage of the tonsil was noted, this process continuing from one to two months. During this period of atrophy the crypts opened and drained, and, in all but a few cases, the exudate disappeared from the throat, and the surface of the tonsils became smooth, pale and of a healthy appearance. With the exception of four cases, no exudate could be squeezed from the deep tonsillar tissue at the end of the period of observation. Later examination of the throat showed the edges of the crypts to be inverted, and in a number of cases white bands resembling scar tissue were noted on the surface.

Other Local Deposits of Lymphoid Tissue.—Another point of as great interest as the shrinkage of the tonsils was the disappearance of the other lymphoid deposits in the throat commonly seen in the form of small nodules on the pillars of the fauces and in masses posterior to the pillars. These deposits are well known as troublesome factors even after extensive surgical removal of the true tonsillar tissue.

Adenoids.—A proportion of the patients treated had, in addition to the tonsil condition, large masses of adenoids.   In order to reduce these a dose of roentgen ray similar to that used on the tonsils was given through the back of the neck.   The results were not as satisfactory as those following the treatment of tonsils, although decided shrinkage was noted in a number of cases.   The lack of uniform results here was doubtless due to the fact that the amount of roentgen ray actually delivered to the tissue after filtration through the bony structures of the head was very small.   It is probable that with a more suitable portal of entry for the rays the adenoids can be influenced to a greater degree.

Bacteriology.—Cultures were made from the crypts of the tonsils and from the nasopharyngeal vault on forty of the forty-six patients before treatment and at intervals afterward.   It is of interest to note that the common organisms found in the throat were unaffected by the treatment, while of the thirty-six cases showing the hemolytic streptococcus and the hemolytic staphylococcus to be present, thirty became free from these organisms by the fourth week after treatment.

COMMENT.

The results reported here suggest the possibility of utilizing the well-known fact that lymphoid tissue is easily destroyed by the roentgen ray for clearing the throat of an excess of this tissue.   In the series reported above, only one patient received more than one treatment.

To judge by our studies on animals, it should be possible to induce almost any degree of atrophy by repeating the roentgen-ray treatments at suitable intervals.   It is possible that the hypertrophied condition may return after a lapse of time; but with the mildness of the roentgen-ray treatment recommended, there is no reason why it should not be repeated as often as desired, with the proper interval between exposures.   The actual amount of roentgen ray used is smaller than that commonly used in the treatment of ringworm of the scalp, from which no bad results have been recorded.

We attribute the disappearance of the hemolytic organisms of the throat, not to the direct action of the roentgen ray on these organisms,

but rather to the proper drainage of the crypts as the tonsil tissue atrophies.

How practicable this treatment will prove can be determined only by the study of a large series of cases followed over a considerable interval of time.

EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

I. TRANSMISSION EXPERIMENTS WITH NASOPHARYNGEAL WASHINGS.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 5 TO 7.

INTRODUCTION.

In planning the present experiments we had in mind the possible presence in the nasopharynx of persons suffering from acute epidemic influenza of some agent the effects of which might be noted in animals. In considering the criteria of activity of this agent we thought, first, of the well known phenomenon in man of leucocytic depression, involving especially the mononuclear cells, during the acute influenzal attack, and next, of changes of a more or less pronounced but possibly transient character, arising in the lungs, which might conceivably predispose to the severe pneumonias that often accompany as a secondary or concurrent infection the influenzal attack.

Furthermore, this study was made during the course of over $1\frac{1}{2}$ years in three successive periods. The first period coincided with the epidemic wave of 1918–19. During this period cases of acute uncomplicated influenza and individuals who had never been affected were studied. The second period embraced the late autumn of 1919, during which influenza did not prevail in New York in epidemic form. During this interepidemic period normal individuals were studied as controls. The third period, the winter of 1920, saw a return of the epidemic. At this time additional cases of the disease were available for investigation. By proceeding in this manner we hoped to check the results for each period against the others. As the sequel will show, we believe that we succeeded in this undertaking, with the consequence that we are enabled to present our findings with perhaps a degree of confidence not otherwise appropriate.

Materials.

The outstanding difficulty in the choice of materials to be employed arose from the necessity of selecting cases of undoubted acute influenza, on the one hand, and of perfectly healthy individuals, who had never suffered from the disease, on the other. In the end the second requirement was more easily fulfilled, as the circumstances of the undertaking admitted of leisurely and painstaking choice of subjects. With uncomplicated influenza, however, the individuals had to be chosen at once, since the epidemic wave of the disease iss notably brief being prolonged chiefly by secondary respiratory infections.

The criteria which were used as guides in the selection of cases of pure influenza were abrupt onset with chilliness, fever, prostration, headache, and muscular pains, especially in the back and limbs. Among the early symptoms were flush and suffusion of the face, injection of the conjunctivæ, soreness of the throat, and harsh, unproductive cough. In the early stages no physical signs were detected in the chest, gastrointestinal symptoms were inconspicuous, and disturbances referable to other internal organs were not complained of or detected by physical examination.

. These symptoms, although striking, were rarely such as could be measured accurately. However, there was one sign that had a quantitative value; namely, the leucocytic picture. Uncomplicated influenza shows a pronounced leucopenia affecting the absolute number of mononuclear cells, chiefly of the lymphocytic variety.[1] This is persistent and even resists at times secondary infectious processes, *e.g.* pneumonia, in which leucocytosis is the rule. As will appear below, great reliance was placed on this quantitative sign in the present experimental studies.

The symptoms and effects endured for from 1 to 3 days, when convalescence, initiated by a lytic fall of temperature, set in, and recovery promptly followed.

[1] The term mononuclears as employed by us includes the leucocytes of the lymphocytic and large mononuclear varieties which have a single homogeneous nucleus. Any indentation of the outline of the nucleus placed the cell in the transitional class, to be counted with the polymorphonuclear cells. Of the varieties of mononuclear cells, the small cells, or lymphocytes, were especially involved in the leucopenia.

Saline washings from the nose and throat were employed. These materials were secured from eight cases of influenza within the first 36 hours of the disease, and from twelve cases at later stages, including the convalescence or the period of postinfluenzal pneumonia. In addition, fourteen individuals who had not been affected were tested during the epidemic or interepidemic period.

Choice of Animal.

In earlier experiments, having in mind a filterable microorganism or virus, we employed *rhesus* monkeys. But this species of animal was found to be unsatisfactory. Monkeys are at best scarce in this country and frequently suffer from pulmonary lesions of a tubercular or other type; the experiments required animals more readily available and free from respiratory affections of any nature. The rabbit was therefore chosen.

EXPERIMENTAL.

Full grown rabbits were used for inoculation, and no rabbit suffering from snuffles or any detectable disease was employed. All animals were subjected to preliminary blood-counting, weighing, and temperature-taking, and any showing variations beyond the average were rejected.[2] These observations were made on 3 to 7 successive days previous to inoculation. The blood counts were carefully controlled; separate apparatus were used for each animal, which was examined throughout the course of observation by the same technician. Particular stress was laid on the total number of leucocytes and the relative and absolute numbers of polymorphonuclear and mononuclear cells.[1] With regard to temperature, it should be emphasized that in these animals the temperature in itself is no indication at times of the extent or absence of pathological involvement but should be interpreted only in conjunction with other findings.

Mode of Inoculation.—The inoculations were made directly into the lungs by means of the intratracheal catheter—a method slightly modified from that employed by Lamar and Meltzer[3]—or by tracheotomy.[4]

The first method, having the advantage of rapidity of operation, consisted in the insertion intratracheally by way of the mouth of a French silk catheter, size 9. An electric otoscope set in the mouth of the animal served as a gag as well as a guide for directing the catheter. The catheter was curved at 90° ½ inch from the tip. In inserting one should avoid slipping over the larynx into the esophagus, thereby contaminating the catheter.

Although tracheotomy requires more time, it is the preferable procedure because contamination by mouth bacteria is avoided. An incision is made directly over the trachea, and a needle, size 19, bent at a right angle, is inserted therein.

Materials Inoculated.—The materials employed for inoculation consisted of (*a*) unfiltered nasopharyngeal washings,[5] (*b*) filtered washings, (*c*) lung tissue suspensions,[6] filtered and unfiltered, from previously inoculated rabbits, (*d*) similar lung tissue preserved in sterile 50 per cent glycerol, (*e*) bacteria and culture materials, and (*f*) control materials. The usual dose for a 2.5 to 3 kilo rabbit was 3 cc. of these materials.

Unfiltered nasal washings were employed in the expectation that they could be purified, or rather deprived of their ordinary bacteria by successive animal passages. It was believed that if this could be accomplished there would be a better chance of preserving and possibly of causing the multiplication of some other variety of microorgan-

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[3] Lamar, R. V., and Meltzer, S. J., *J. Exp. Med.*, 1912, xv, 133.

[4] With either method light ether anesthesia was given.

[5] Nasopharyngeal washings were obtained as follows: The patient's mouth was rinsed with warm saline solution. Then each nasal cavity was washed with 25 to 35 cc. of saline solution, the washings being returned by way of the mouth and collected in a sterile container. The entire fluid was shaken with glass beads in a mechanical shaker for 15 minutes at high speed, or until a homogeneous mixture resulted.

[6] Lung tissue was prepared for inoculation as follows: The selected portion of the lungs was chopped and then ground with sterile, fine, white sand, and a proportional amount of saline solution was added in the ratio of 20 cc. of saline solution to the two entire lungs. The suspension was then centrifuged at low speed and the clearer supernatant fluid employed for inoculation.

ism, more resistant and virulent perhaps, which would give to the washings from cases of uncomplicated influenza a quality lacking in others. If was, of course, realized that not in every instance could this favorable outcome be looked for. Now and then it was to be expected that a virulent pneumococcus or streptococcus would set up a pneumonia to which the animal would succumb. But if the ordinary bacteria could be suppressed by animal passages in a few instances and something survive which produced definite changes in the tissues of the rabbits—the blood and lungs, for example—the washings from cases of influenza might thus be distinguished in their effects from the washings of another origin. In this way the operation of a pathogenic agent is to be deduced, although it might not be possible to determine certainly that this agent is the inciting microbic agent of influenza. However, if a certain correspondence in tissue and other effects can be shown to exist between the individual suffering from influenza and the rabbit inoculated with materials originally derived from influenza cases and free from all ordinary bacteria, an idea as to the probable nature of the pathogenic agent is gained which encourages further investigation along the indicated lines.

Inoculation of Unfiltered Washings.

There were inoculated into the lungs of rabbits the unfiltered nasopharyngeal secretions derived from five cases of uncomplicated influenza during the first epidemic, and three during the second, in the first 36 hours of the disease, and from eleven cases during the first epidemic and one during the second in the later stages of the affection. The following effects were induced by the materials from seven of the eight early cases, but not by any from the twelve cases examined after 36 hours.

Clinical Effects.—From 24 to 48 hours after inoculation fever developed, associated with the ordinary signs of indisposition in a rabbit, such as listlessness and ruffled hair, and conjunctivitis. The striking feature, however, was the definite and often marked leucopenia resulting from depression of the mononuclear cells, as shown in Text-fig. 1, *a*, *b*, and *c*. If the condition was allowed to run its natural course, these symptoms endured for about 3 days, the animal then returning to

normal. If the rabbit was killed—for in the absence of infection by ordinary bacteria none died—an unusual pathological picture was revealed.

Pathological Effects.—The respiratory organs were affected to the exclusion of all others. No pleuritis or exudate in the pleural cavity was evident. The lungs were voluminous as a result of edema and emphysema and had a mottled hemorrhagic appearance. The hemorrhages on the surface, beneath the pleura, were diffuse or discrete, occupying areas a few millimeters in extent or covering a large part of a lobe. In addition, minute petechiæ were seen scattered over the entire surface. On section of the lungs the cut surface revealed a hemorrhagic edema; it dripped a blood-stained, frothy fluid. The hemorrhages again were either diffuse and large, or discrete and small, in the latter instance being numerous.

On microscopic section carried through various parts of the lungs the lesions were found to consist (*a*) of hemorrhagic foci, and (*b*) of edema and emphysema. The hemorrhages varied in size in accordance with the observed macroscopic appearance, some being microscopic in nature. The edema was more extensive than the hemorrhages and involved alveoli and interalveolar strands of tissue. The alveoli contained coagulated serum or red corpuscles, mononuclear cells, and also at times polymorphonuclear cells of eosinophilic type and desquamated epithelial cells. The interalveolar strands were infiltrated with mononuclear cells and large cells the foreign nature of which was not always clear. Fibrin was sometimes present in small amounts. The bronchi, also, were at times filled with erythrocytes, exfoliated and degenerated epithelia, and leucocytes. The capillaries were distended with blood.

No ordinary bacteria were seen in impression films of the lung tissue or in sections stained by Gram's or MacCallum's[7] method, or in aerobic or anaerobic cultures of the tissue.

The two following protocols are presented in order to show the clinical and pathological effects, regarded as typical, which arise independently of the presence of ordinary bacteria of any demonstrable kind.

[7] MacCallum, W. G., The pathology of the pneumonia in the United States Army camps during the winter of 1917–18, Monograph of The Rockefeller Institute for Medical Research, No. 10, New York, 1919, 47.

PROTOCOL 1.

Patient 8.—M. T., adult female. Feb. 9, 1919. Onset sudden with chills and fever. Feb. 10. Photophobia; prostration; muscular pains in the back and extremities; mild unproductive cough. Temperature 37.8°C.; pulse 80. Leucocytes 8,725, of which 1,832 were mononuclears. Physical examination showed lungs to be clear; pharynx red and congested; conjunctivæ injected. No other organs affected. Cultivation of sputum and nasopharyngeal washings yielded Pneumococcus Type IV; no Pfeiffer bacilli. The fever persisted for 48 hours and was followed by an uneventful recovery.

Animal Inoculation.—The unfiltered nasopharyngeal washings obtained 20 hours after the onset of the symptoms were inoculated intratracheally into Rabbit A.[8]

First Passage. Rabbit A.—Before injection the normal blood counts showed the following results: Feb. 8, 1919. Leucocytes 13,050, of which there were 6,525 each of polymorphonuclears and mononuclears. Feb. 10. Prior to inoculation, leucocytes 14,425, of which 7,357 were polymorphonuclears and 7,068 mononuclears. Temperature 39.5°C. Injected intratracheally with 3 cc. of the nasopharyngeal washings from Case 8. Feb. 11. Conjunctivitis. Leucocytes 12,550, of which 7,781 were polymorphonuclears and 4,769 mononuclears. Temperature 39.6°C. Feb. 12. Conjunctivitis persists. Leucocytes 11,450, of which 7,786 were polymorphonuclears and 3,664 mononuclears. Temperature 39.8°C.

The development within 24 hours of the conjunctivitis, the rise in temperature with depression of the leucocytes and mononuclears, which endured for 48 hours, were indications for killing the animal. Hence on Feb. 12 the animal was killed.[9]

Autopsy.—The lung condition was such as has been described as a typical effect of the inoculation.

Aerobic Cultures.—Cultures on the usual media remained sterile.[10]

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[8] Usually more than one rabbit was inoculated at the same time, but in this instance only the course of the rabbit used for further transmission experiments is described.

[9] In all experiments the animal was killed by a sharp blow, which dislocated the upper cervical vertebræ. In this way the complicating effects of ether anesthesia on the respiratory tract were avoided. The blow should be properly directed; otherwise the chest may be struck, thus causing contusion of the lung, or the skull may be broken with consequent profuse hemorrhages and aspiration of the blood, in which event the pathological picture is obscured.

[10] As a routine practice aerobic blood cultures were made before the animal was killed. Also pieces of lung tissue were planted in 1 per cent dextrose broth, and in this medium plus rabbit blood. Anaerobic cultures of lung tissue will be described in another communication.

Second Passage. Rabbit B.—Average normal leucocyte count before inoculation 10,775, of which 5,604 were mononuclears. Feb. 12, 1919. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit A. Feb. 13. Leucocytes 7,600, of which 3,268 were mononuclears. Feb. 14. Killed.

Autopsy.—The lung appearance was typical.

Aerobic Cultures.—Remained sterile.

Third Passage. Rabbit C.—Average normal leucocyte count before inoculation 15,060, of which 7,461 were mononuclears. Feb. 14, 1919. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit B. Feb. 15. Leucocytes 5,675, of which 2,043 were mononuclears. Feb. 16. Leucocytes 8,760, of which 3,942 were mononuclears. Killed.

Autopsy.—Typical lung lesions, but to a milder degree.

Aerobic Cultures.—No growth of ordinary bacteria obtained.

A suspension of the lung tissue from this rabbit failed, however, to produce a similar effect in the succeeding rabbit. At this point the series of transmissions was terminated.

PROTOCOL 2.

Patient 16.—N. L., adult female. Mar. 28, 1919. Onset sudden with chills and fever. Mar. 29. Temperature 39°C.; muscular pains in back; prostration and weakness; epistaxis; unproductive cough; coryza and photophobia. Flushed face; injected conjunctivæ; congested pharynx; lungs, a few râles anteriorly and posteriorly on left side only. No other organs affected. Leucocytes 7,300; mononuclears 2,117. Cultivation of sputum and nasopharyngeal washings yielded Pneumococcus Type IV. Sputum injected into mouse yielded no Pfeiffer bacilli, only the pneumococci. On the 2nd day of illness the temperature declined, and the general condition improved. On the 3rd day a relapse occurred, but recovery began on the 4th day.

Animal Inoculation.—The unfiltered nasopharyngeal washings were obtained 36 hours after the onset and were inoculated into the lungs of Rabbit A.

First Passage. Rabbit A.—Mar. 29, 1919. Injected intratracheally with 3 cc. of the nasopharyngeal washings from Case 16. Mar. 30. Conjunctivitis. Leucocytes decreased from the norm of 8,200 to 6,700, and mononuclears from 4,100 to 2,680. Killed.

Autopsy.—Lungs showed the typical lesions.

Aerobic Cultures.—Free from growth.

Second to Sixth Passages. Rabbits B, C, D, E, and F.—Each rabbit was injected similarly with a suspension of the lung tissue of the immediately preceding rabbit of the series. All showed uniform clinical effects. After 24 to 48 hours there developed conjunctivitis, leucopenia, mononuclear depression, and varying temperature reactions. None died, but all were killed 48 hours after the injection, except Rabbit C which was killed after 24 hours.

Autopsy.—There were present varying degrees of the typical lung lesions. The lung lesions of the last (sixth) rabbit passage are shown in Figs. 1, 3, and 4.

Aerobic Cultures.—In each instance free from growth.

Further transmissions were not made and hence this series terminated in the sixth rabbit passage.

The two series of experiments recorded in Protocols 1 and 2 indicate that definite and consistent clinical and pathological effects were induced in two series of rabbits with materials derived from the nasopharynx of recent acute cases of influenza which were independent of the presence of ordinary aerobic bacteria.

The next series of three protocols is given in order to bring out the fact that the clinical and pathological effects regarded as typical appear even when ordinary bacteria are cultivable, and also that these bacteria are suppressible through successive inoculation while the typical effects continue to occur.

<div align="center">PROTOCOL 3.</div>

Patient 6.—K., adult female. Jan. 27, 1919. Onset sudden with chills and fever. Jan. 28. Pains in the muscles of back and legs; prostrated and weak; severe frontal headache; mild unproductive cough. Temperature 38°C. Physical examination showed no signs in chest; conjunctivæ injected. Cultivation of sputum and nasopharyngeal washings yielded Pfeiffer bacilli and Pneumococcus Type IV. Symptoms persisted for 3 days, followed by an uneventful recovery.

Animal Inoculation.—The unfiltered nasopharyngeal washings obtained 24 hours after the onset of the symptoms were inoculated into the trachea of Rabbit A.

First Passage. Rabbit A (Text-Fig. 1, a).—Jan. 28, 1919. Injected intratracheally with 3 cc. of the nasopharyngeal washings from Case 6. Blood count of rabbit prior to inoculation showed 15,025 leucocytes, of which 8,414 were mononuclears. Jan. 29. Purulent conjunctivitis. Leucocytes 6,600, of which 2,244 were mononuclears. Jan. 30. Leucocytes 8,025, of which 2,006 were mononuclears. Killed.

Autopsy.—Left lung showed gray hepatization with fibrinous pleuritis (lobar pneumonia); right lung, aside from a small area of atelectasis in upper lobe, showed edema and emphysema with large numbers of petechial hemorrhages.

Aerobic Cultures.—Left lung yielded a growth of Pneumococcus Type IV, avirulent for mice;[11] right lung yielded no growth of ordinary bacteria.

The right lung was employed for inoculation into the next rabbit.

Second Passage. Rabbit B (Text-Fig. 1, b).—Jan. 30, 1919. Injected intratracheally with 3 cc. of the suspension of tissue from right lung of Rabbit A.

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[11] The significance of the ordinary bacteria encountered in these experiments will be dealt with in another communication.
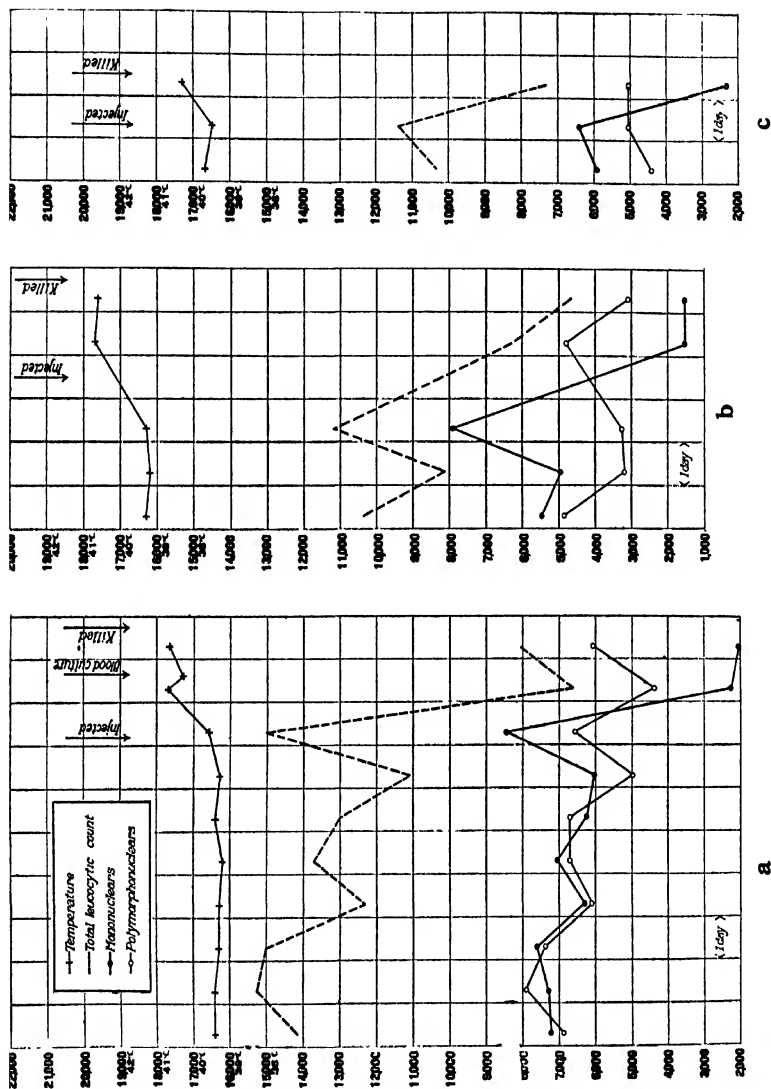
TEXT-FIG. 1, *a*, *b*, and *c*. Effect on the blood count and temperature. The rise in temperature and the depression in the total white blood cell count caused by a deficiency of mononuclears are shown. (*a*) First rabbit passage of the nasopharyngeal washings from a case in the early stage of uncomplicated influenza (Patient 6). (*b*) Second rabbit passage from the same case. (*c*) Seventh rabbit passage from Patient 11.

Feb. 1. Killed after having shown a depression of 6,765 in the total count and 6,269 in the mononuclear count on the 2nd day after injection.

Autopsy.—Both lungs were edematous and emphysematous and showed multiple punctate hemorrhages.

Aerobic Cultures.—No growth obtained from heart's blood or lung tissue.

Third Passage. Rabbit C.—Feb. 1, 1919. Normal leucocyte count before inoculation was 10,950, of which 5,585 were mononuclears. Temperature 39.1°C. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit B. Feb. 2. Both conjunctivæ injected. Leucocytes 9,300, of which 3,255 were mononuclears. Temperature 39.7°C. Feb. 3. Conjunctivitis. Leucocytes 7,350, of which 2,850 were mononuclears. Temperature 40.5°C. Killed.

Autopsy.—Lungs showed typical hemorrhagic edema and emphysema.

Aerobic Cultures.—No growth.

Fourth Passage. Rabbit D.—Feb. 3, 1919. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit C. On the next 2 days the temperature rose from 39.3° to 39.5° and 40°C. Conjunctivitis was present with a mononuclear depression of 2,455 cells on the 1st day and 1,829 on the 2nd. Feb. 5. Killed.

Autopsy.—Besides a few areas of consolidation at the base measuring 5 mm. in diameter, the lungs showed edema, patches of emphysema, and multiple punctate hemorrhages.

Aerobic Cultures.—Heart's blood, no growth; lung tissue, lower lobes Pneumococcus Type IV, upper lobes no growth.

The upper lobes were employed in the next transmission experiment.

Fifth Passage. Rabbit E.—Feb. 5, 1919. Injected intratracheally with 3 cc. of a suspension of tissue from the upper lobes of lungs of Rabbit D. During the next 2 days the temperature rose from 39.5° to 40.4° and 39.8°C. Leucopenia was noted, with a mononuclear depression from 5,377 to 2,030 on the 1st day and 3,468 on the 2nd. Feb. 7. Killed.

Autopsy.—Lungs showed the typical lesions similar to those of Rabbit B.

Aerobic Cultures.—No growth.

Sixth Passage. Rabbit F.—The effects were an exact repetition of those of Rabbit E so that it is unnecessary to give them in detail.

Aerobic Cultures.—No growth.

Seventh Passage. Rabbit G.—Feb. 9, 1919. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit F. On the 2 following days the temperature rose from 39.6° to 39.8°C. Leucopenia developed, the count diminishing from 15,640 to 12,075 and 10,725 respectively, with a mononuclear depression from 8,289 to 2,415 and 3,325 cells. Conjunctivitis. Feb. 11. Killed.

Autopsy.—Lungs showed typical lesions similar to those of Rabbits B, C, E, and F.

Aerobic Cultures.—No growth.

Eighth Passage. Rabbit H.—Feb. 11, 1919. Temperature 39.7°C. Leucocytes 12,560, of which 5,275 were mononuclears. Injected intratracheally with

3 cc. of the suspension of lung tissue from Rabbit G. Feb. 12. Leucocytes 3,250, of which 2,015 were mononuclears. .Temperature 40.35°C. Feb. 13. Leucocytes 4,300, of which 2,193 were mononuclears. Temperature 40.6°C. Died.

Autopsy.—Right lung showed consolidation and fibrinous pleuritis; left lung revealed typical hemorrhagic edema with emphysema.

Aerobic Cultures.—Left lung, no growth; right lung, *Micrococcus catarrhalis.*

The left lung was employed in the next transmission experiment.

Ninth Passage. Rabbit I.—Feb. 13, 1919. Leucocytes 11,200, of which 5,488 were mononuclears. Temperature 39.2°C. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit H. On the next 2 days the temperature rose to 39.6° and 39.8°C., the leucocytes diminished to 8,900 and 8,675 cells, and the mononuclears to 3,382 and 2,776 respectively. Feb. 15. Killed.

Autopsy.—Lungs showed the typical lesions.

Aerobic Cultures.—No growth.

Tenth Passage. Rabbit J.—Feb. 15, 1919. Injected with lung tissue from Rabbit I with similar results.

Eleventh Passage. Rabbit K.—Feb. 17, 1919. Injected similarly with lung tissue from Rabbit J. For 3 days the total leucocyte count was diminished from 10,120 to 8,320, 7,125, and 7,975, and the mononuclear count from 4,250 to 2,573, 2,494, and 1,674 respectively. Allowed to recover for experiment on immunity.

This series, therefore, was voluntarily terminated in the eleventh rabbit passage. It seems certain that it could have been continued for some time and possibly indefinitely. Besides what were regarded as typical results, this series included pneumonic infections with Pneumococcus Type IV and *Micrococcus catarrhalis* in the first, fourth, and eighth passages. Fortunately the pneumonic areas were restricted and did not lead to infection of both lungs. Hence the successive inoculation of the bacteria-free lung tissue could be continued. The death of Rabbit H of the eighth passage was the first to occur in our experiments.

PROTOCOL 4.

Patient 11.—R. N., adult male. Feb. 18, 1919. Onset sudden with chills and fever. Feb. 19. Temperature 39°C.; pulse 80. Prostration; general malaise; coryza and photophobia; unproductive cough. Physical examination showed conjunctivitis and congested pharynx; right lung, no signs; left lung, at base, few indefinite râles. Leucocytes 5,040, of which 1,764 were mononuclears. Cultures from sputum and nasopharyngeal washings yielded only *Streptococcus viridans.* Symptoms endured for 3 days, followed by an uneventful recovery.

Animal Inoculation.—The unfiltered nasopharyngeal washings obtained 24 hours after the onset of the symptoms were inoculated into the lungs of Rabbit A.

First Passage. Rabbit A.—Feb. 19, 1919. Leucocytes 13,960, of which 8,794 were mononuclears. Temperature 39.3°C. Injected intratracheally with 3 cc. of the nasopharyngeal washings from Case 11. Feb. 20. No change. Feb. 21. Conjunctivitis. Temperature 40.5°C. Leucocytes decreased to 6,100; mononuclears to 2,989. Killed.

Autopsy.—Lungs showed the typical lesions.

Aerobic Cultures.—No growth.

Second Passage. Rabbit B.—Feb. 21, 1919. Leucocytes 12,650, of which 6,451 were mononuclears. Temperature 39.7°C. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit A. On the next 2 days there were fever (41.3° and 40.6°C.), leucopenia (leucocytes 5,760 and 5,775), and mononuclear depression to 2,592 and 1,098 cells. Feb. 23. Killed.

Autopsy.—Lungs showed the typical lesions.

Aerobic Cultures.—No growth.

Third to Eighth Passages. Rabbits C, D, E, F, G, and H.—These rabbits developed, 24 hours after the intratracheal injection of the suspensions of lung tissue from the immediately preceding rabbit in the series, a pronounced leucopenia (in one case a decrease in leucocytes from 15,900 to 2,350), mononuclear depression (in Rabbit D, for example, from 7,632 to 940 cells), conjunctivitis, and fever (usually above 40.2°C.). The rabbits of the third, fourth, and fifth passages died 48 hours after injection; those of the sixth, seventh (Text-fig. 1, *c*), and eighth were killed in the usual manner.

Autopsy.—In all instances definite areas of consolidation were found in one or more lobes, while elsewhere the lung contained the typical areas of hemorrhage and edema accompanied with emphysema.

Aerobic Cultures.—The consolidated foci of the third, fourth, and fifth passages yielded Pneumococcus Type IV, and of the sixth, seventh, and eighth an atypical Type II pneumococcus.

Ninth Passage. Rabbit I.—Mar. 5, 1919. Leucocytes 14,225, of which 7,397 were mononuclears. Temperature 39.5°C. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit H. After 48 hours the temperature rose to 40°C., the leucocytes diminished to 10,225, of which 6,237 were mononuclears. This persisted for 2 days. Mar. 9. Killed.

Autopsy.—Lungs showed the typical lesions.

Aerobic Cultures.—No growth.

Tenth Passage. Rabbit J.—Average leucocyte count prior to inoculation was 15,353, of which 7,587 were mononuclears. Temperature 39.6°C. Mar. 9, 1919. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit I. After 24 hours, and for the next 3 days, the temperature rose to 40°C., the leucocytes diminished to 11,400, reaching 8,800 on the 3rd day, and the mononuclears decreased to an average of 4,886 cells. Mar. 13. Killed.

Autopsy.—Lungs showed the typical lesions.

Aerobic Cultures.—No growth.

With this, the tenth rabbit passage, the series was discontinued.

This series closely reproduces the previous one. The transmission experiments were discontinued with the tenth passage and at a time when the typical effects were still being produced regularly. Several instances of fatal intercurrent infection were encountered, the pneumonia present being associated with Pneumococcus Type IV or atypical Type II. But other portions of the lungs, which were free of these and other ordinary bacteria, were suitable for the transmission experiments.

PROTOCOL 5.

Patient 17.—S. C., adult male. Apr. 10, 1919. Sudden onset at night with symptoms of languor, chills, headache, and dull pain in the back. Apr. 11. General malaise; prostration; fever; headache; no cough or rhinitis. Physical examination showed conjunctivæ injected, pharynx congested, and lungs clear (also by radiographic examination). Other organs unaffected. Leucocytes 5,875, of which 2,059 were mononuclears. Blood culture sterile. Sputum not obtainable. A throat culture yielded no Pfeiffer bacilli on oleate agar (Avery medium[12]), but a Gram-negative coccus on blood agar plates. Nasopharyngeal washings yielded this coccus and *Streptococcus viridans*. On the 3rd day of illness the temperature was normal, and an uneventful recovery followed.

. *Animal Inoculation.*—The unfiltered nasopharyngeal washings obtained 12 hours after the onset of symptoms were inoculated intratracheally into Rabbit A.

First to Third Passages. Rabbits A, B, and C.—These animals were employed for the first, second, and third passages respectively, the same methods being used as those described in the previous transmission experiments. Rabbit A died over night, Rabbit C after 2 days, and Rabbit B was killed. In all instances fever (above 40°C.) developed, with conjunctivitis and a prompt and marked leucopenia and mononuclear depression similar to those already given.

Autopsy.—The lungs revealed typical hemorrhagic edema, the hemorrhages varying from diffusely scattered small foci to large areas involving almost an entire lobe, sometimes of infarct shape. There was no definite consolidation.

Aerobic Cultures.—The lungs of the three animals yielded Type IV pneumococci.

Fourth to Fifteenth Passages.[13]—These were initiated with a filtrate, free from aerobic bacteria, of the suspension of lung tissue from Rabbit C.

In this experiment the pneumococcus present in the nasopharyngeal secretions was not suppressed during the first to third rabbit passages.

[12] Avery, O. T., *J. Am. Med. Assn.*, 1918, lxxi, 2050.

[13] These experiments will be described in detail in a later communication.

But the effects in the animal, even though severe and fatal, bore so close a resemblance to those arising in the four previous series of rabbits that it was deemed advisable to free the material from the pneumococcus by filtration. The filtrate thus secured, free from aerobic organisms, was passed through a series of twelve animals in which the typical clinical and pathological effects were obtained.

Thus far we have dealt with cases of influenza which occurred during the epidemic of 1918–19. The next series of experiments relates to cases arising in the winter of 1920 of which three uncomplicated cases in the early stage of the affection were available for study.

PROTOCOL 6.

Patient 24.—H. J., adult female. Jan. 20, 1920. Onset sudden with chills and fever. Jan. 21. Prostration; photophobia; muscular pains; unproductive cough. Conjunctivitis and injected pharynx; lungs negative. Leucocytes 2,800, of which 336 were mononuclear. Cultures from the nasopharyngeal washings yielded numerous colonies of *Staphylococcus albus* and *Micrococcus flavus*, and a moderate number of Pfeiffer bacilli.

Animal Inoculation.—The filtered and unfiltered washings obtained 20 hours after the onset were inoculated into the lungs of three rabbits which, within 24 hours, developed conjunctivitis, fever, leucopenia, and mononuclear depression similar to those in the other instances cited. The animals were killed 48 hours after inoculation.

Autopsy.—The lungs showed the typical hemorrhagic, emphysematous, and edematous condition.

With the lungs of these rabbits another series of transmissions, consisting of three rabbits, two for the filtered material, to be described in another communication, and one for the unfiltered, was carried out. The results in each case were typical.

PROTOCOL 7.

Patient 26.—M. O., adult female. Feb. 4, 1920. Onset sudden with chills and fever. Temperature 40.1°C. Feb. 5. Fever; muscular pains; unproductive cough. Conjunctivitis; lungs clear. Leucocytes 6,000, of which 1,380 were mononuclears. Cultures from the nasopharyngeal washings yielded mainly *Staphylococcus albus* and also pneumococci, hemolytic streptococci, and occasional Pfeiffer bacilli.

Animal Inoculation.—The filtered and unfiltered nasopharyngeal washings obtained 30 hours after the onset were injected intratracheally into Rabbits A, B, and C, the last two receiving the filtrates. These animals showed the typical clinical and pathological effects, similar to those of the rabbits described in Protocols 1 and 2. No ordinary bacteria were detected in cultures or films of the lungs. The rabbits were killed 48 hours after the inoculation.

With the lungs of these animals another series of transmissions was carried out, with results which were regarded as typical. The gross appearance of the lungs of one rabbit is shown in Fig. 2. The lungs of one rabbit of the first passage of the filtered material (Rabbit C) were preserved in sterile 50 per cent glycerol for 4 months, and the lungs of Rabbit D, the second passage of the unfiltered washings, for 10 days. With these glycerolated lungs further transmissions were carried out to be described in another communication, until the experiment was voluntarily discontinued with the sixth rabbit passage. All showed the typical clinical and pathological effects, and in no instance were ordinary bacteria detected in cultures or in stained films from the lungs.

PROTOCOL 8.

Patient 27.—P. S., adult male. Feb. 9, 1920. Onset sudden with chills and fever. Feb. 10. Fever; muscular pains; sore throat; tracheal cough; headache; prostration. Lungs clear. Leucocytes 13,000, of which 10,140 were polymorphonuclears. Cultivation of nasopharyngeal washings yielded an almost pure culture of hemolytic streptococci and a few colonies of Pfeiffer bacilli. The question was raised of a complicating streptococcus sore throat in this patient.

Animal Inoculation.—The filtered and unfiltered nasopharyngeal washings obtained 20 hours after onset were injected into the lungs of five rabbits. The filtrates produced no effect; the unfiltered material caused abscess of the lung, from which hemolytic streptococci were isolated. The material was not inoculated further.

It is seen that the nasopharyngeal secretions of two of three cases of influenza during the second or 1920 epidemic gave rise in rabbits to clinical and pathological effects, independently of the presence of ordinary bacteria, similar to those obtained during the first or 1918 epidemic.

Negative Transmission Experiments.

In addition to the positive transmission experiments presented under Protocols 1 to 8, several cases of influenza, at somewhat later stages in the evolution of the disease, were studied in the same manner. The cases from the 1918 epidemic included five which were in the 3rd day of the disease when the washings were secured, four in the afebrile and convalescent stage, and two in course of a secondary pneumonia. From the 1920 epidemic one case only, on the 1st afebrile day, was studied.

The washings from the nasopharynx of these individuals were collected and injected into rabbits in the manner already described, and

in none of them was the characteristic effect on the blood count observed (Text-fig. 2). When a blood change did occur it was of the nature of a polymorphonuclear leucocytosis. The lung lesions, when any were present, consisted of lobar consolidation in which pneumococci, as a rule, were demonstrated.
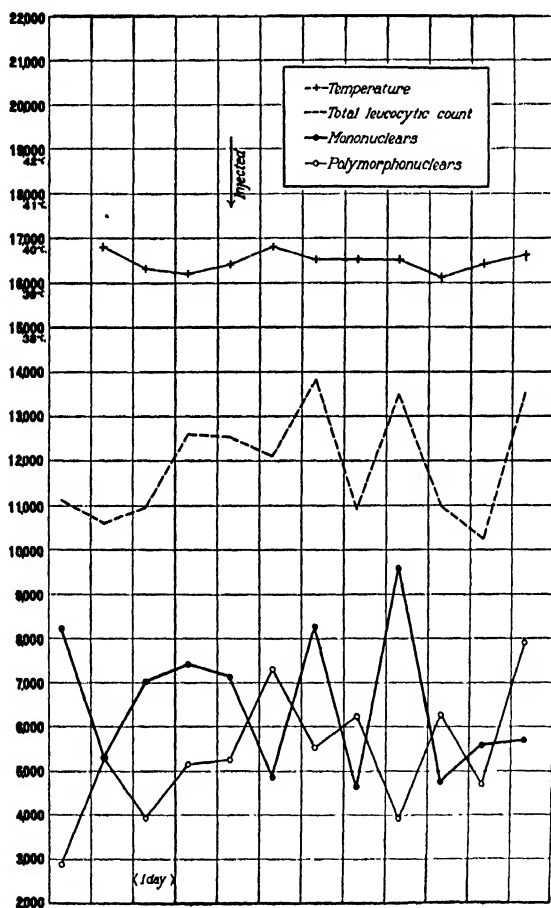
TEXT-FIG. 2. First rabbit passage of the nasopharyngeal washings from Patient 5. The washings were obtained at the beginning of the 3rd day of uncomplicated influenza. No effect on blood count and temperature.

Control Experiments (Text-Fig. 3, a and b).

The control tests consisted of the injection into the lungs of rabbits of saline solution, suspensions of normal rabbit lungs, normal rabbit serum, foreign protein, such as human ascitic fluid, bacteria of the

ordinary species,[14] including Pfeiffer's bacillus and its poison as prepared by Parker's method,[15] and finally, the nasopharyngeal secretions from fourteen persons free from influenza and tested in the epidemic
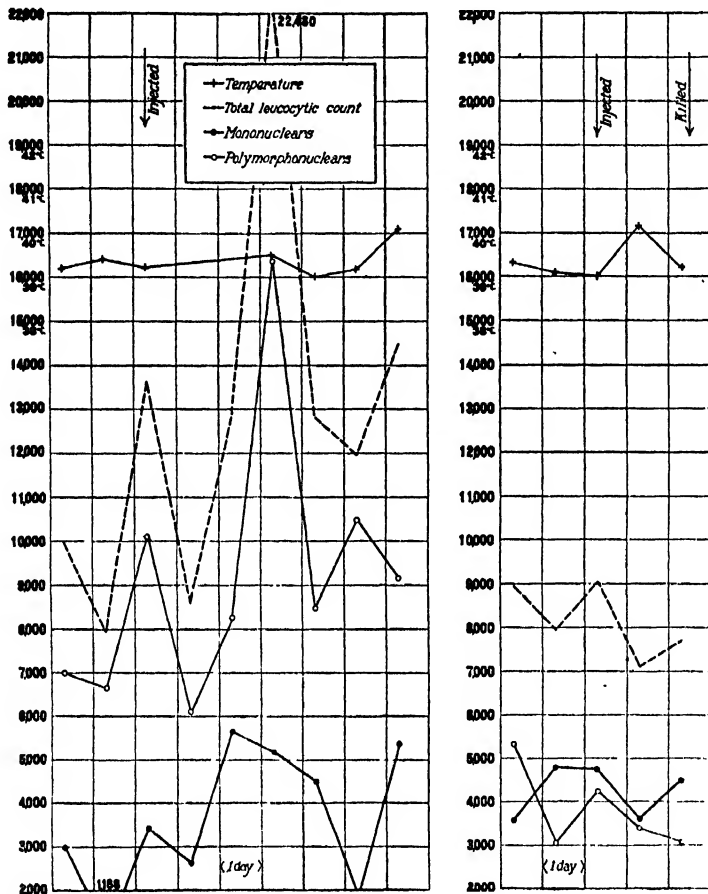
TEXT-FIG. 3, *a* and *b*. Effect on the blood count and temperature of intratracheal inoculations of control materials. (*a*) Inoculation of nasopharyngeal secretions from a normal individual free from an influenzal attack. A transient polymorphonucleosis on the 3rd day after inoculation is shown. (*b*) Inoculation of a suspension of normal rabbit lung. No effect on blood count and temperature.

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[14] Control experiments with ordinary bacteria injected intratracheally will be described in another communication.

[15] Parker, J. T., *J. Immunol.*, 1919, iv, 331.

and interepidemic periods. Of the latter, seven suffered from early or later stages of coryza. The lung tissue of the inoculated rabbits was in turn reinoculated into two successive series of rabbits. None of the 55 animals inoculated with the control materials or these secretions showed the familiar clinical and pathological action; a few gave a polymorphonucleosis with frank lobar pneumonia, others a mononucleosis without lung involvement, and still others inconstant effects.

<div align="center">DISCUSSION.</div>

The object of the present investigation was to determine, if possible, whether the secretions of the nasopharynx of individuals suffering from epidemic influenza exhibited on inoculation into animals any peculiarities of action or properties which would serve to distinguish them from the secretions of individuals not so affected. Obviously, the first requisite was a standard by means of which this action, or effect, could be detected. We sought one which was subject to measurement with at least a fair degree of accuracy and did not necessitate killing the inoculated animal. This criterion was found in the blood, associated with changes in the absolute and differential white blood count and correlated with the leucocytic curve in uncomplicated cases of epidemic influenza in man. A second criterion, observable after the animal had been killed, was discovered in certain hemorrhagic, edematous, and emphysematous changes in the lungs.

The next point was the determination of the relation of the changes noted in the leucocyte count and the lung structures to the ordinary bacterial flora of the nasopharynx. The fact that the changes were regularly observable, under favorable conditions, in the complete absence of ordinary bacteria in culture and in film, was regarded as particularly significant.

The deduction arrived at, therefore, as a result of the experiments carried out in rabbits was to the effect that patients with epidemic influenza, within at least the early stage of the obvious infection, carry in their nasopharyngeal secretions a substance which is not ordinary bacteria or their metabolic products. This substance when inoculated intratracheally into rabbits readily causes fever, leucocytic and particularly mononuclear cell depression, lung hemorrhage, edema, and em-

physema. Whatever this active substance is, it seems to disappear from or to diminish in the nasopharyngeal secretions of cases of epidemic influenza so as to be no longer discoverable by inoculation tests about 36 hours after the obvious symptoms of the disease have appeared, and to be absent from healthy persons and in other pathological conditions.

When implanted in the lungs of the rabbit this substance appears to increase, since it remains active through a long series of inoculations of the lung tissue in successive passages through rabbits.

The substance is readily filterable through Berkefeld filters. It is capable of surviving and apparently of multiplying in association with ordinary bacteria not only in the nasopharyngeal secretions in man but also in the lungs of rabbits, in the latter at least for a time.

When the unfiltered washings containing nasopharyngeal secretions of patients in early stages of epidemic influenza are injected intratracheally into rabbits, this substance when present exerts its peculiar action while some multiplication of ordinary bacteria (usually Pneumococcus Type IV) is going on in the lung. The successive passage of unfiltered emulsions of selected parts of the lungs, away from obviously infected and consolidated areas, leads often to rapid and complete disappearance of the ordinary bacteria and survival and possibly increase of this active substance.

No attempt will be made in this paper to define further the nature of the active substance or to relate it more accurately and specifically with the etiology of epidemic influenza.

SUMMARY.

An active substance has been detected, by the methods described, in five patients in early stages of epidemic influenza during 1918–19 and two patients in early stages of epidemic influenza during 1920. It was not detected in twelve cases of the same disease in which the onset of obvious symptoms occurred more than 36 hours before washing of the nasopharynx was carried out, nor was it found in the secretions of fourteen individuals free from the syndrome of influenza either during the epidemics or the interval between them.

With this substance a clinical and pathological condition has been induced in rabbits, affecting the blood and pulmonary structures

mainly, which could be maintained and carried through at least fifteen successive animals. For this reason, and also because of the dilution between passages, we are led to believe that we were dealing with the actual transmission of a multiplying agent rather than with a passive transference of an original active substance.

In some of the experiments secondary infections by ordinary bacteria were encountered. The relation of these microorganisms to this active substance will be dealt with fully in another communication. However, the essential effects were produced by a substance wholly unrelated to these bacteria.

The similarity that exists between the effects produced in rabbits on the blood and the lungs and those occurring in man in epidemic influenza provides a basis for further investigation on the inciting agent of epidemic influenza.

EXPLANATION OF PLATES

PLATE 5.

Fig. 1. Gross lesions of a lung from Rabbit F, representing the sixth rabbit passage of the nasopharyngeal secretions from Patient 16. The hemorrhages, edema, and emphysema of the lung, more marked in the upper lobe, and absence of pneumonic consolidation are noteworthy. Natural size.

Fig. 2. Gross lesions of the lungs from Rabbit D, representing the second rabbit passage of the nasopharyngeal secretions from Patient 26. This case occurred in the second epidemic of 1920 and is to be compared with Fig. 1 derived from the first epidemic of 1918–19. Hemorrhages, edema, and emphysema are shown. A small area of atelectasis is seen at the inner margin of the lower right lobe. Natural size.

PLATE 6.

Fig. 3. Microscopic appearance of a section of the lung shown in Fig. 1. Edema and emphysema are present. A vessel is shown distended with blood, and mononuclears may be seen in the interalveolar tissues. × about 190.

PLATE 7.

Fig. 4. A different section of the same lung. The small discrete hemorrhages, the edema, and the cellular exudate are shown. × about 190.

Fig. 1.

Fig. 2.

(Olitsky and Gates · Nasopharyngeal secretions from influenza. I.)
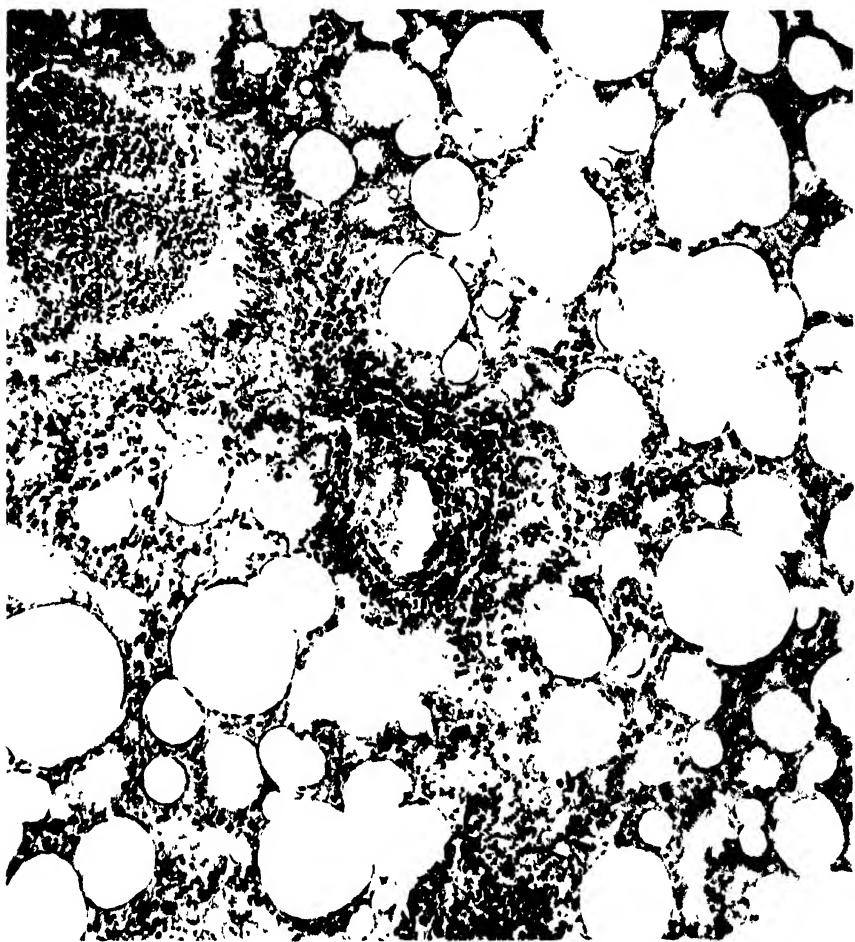
Fig. 3.
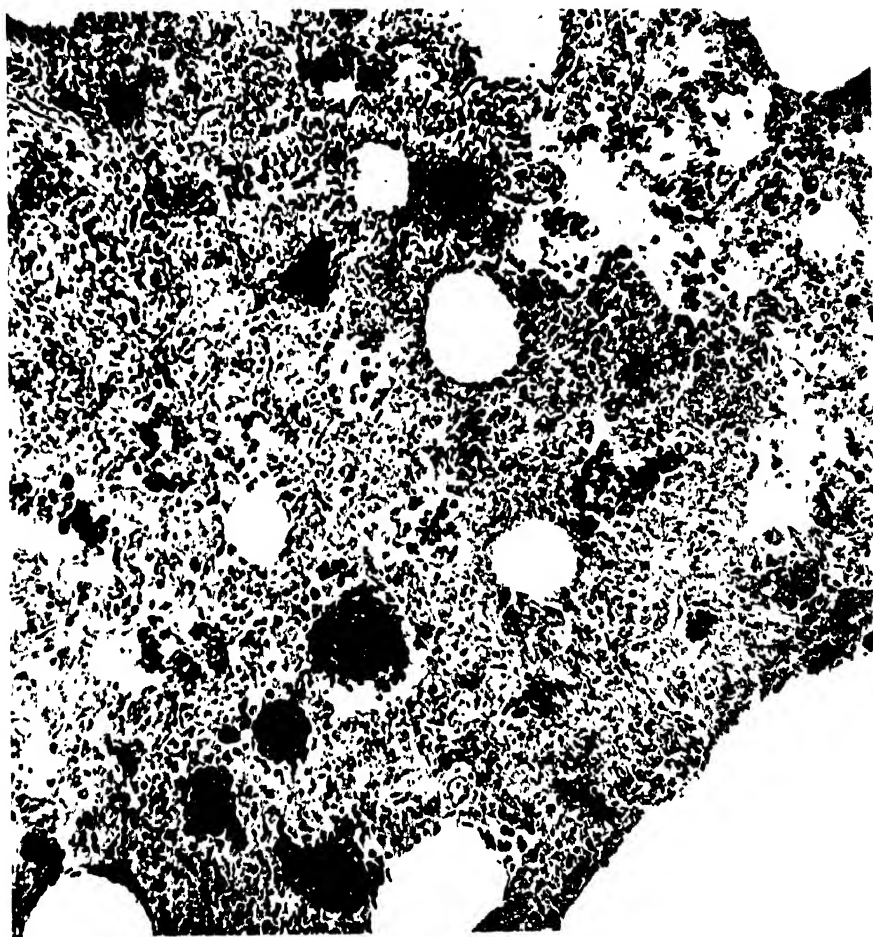
Fig. 4.

EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

II. FILTERABILITY AND RESISTANCE TO GLYCEROL.

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PLATES 32 TO 34.

In our first communication[1] we described the particular effects induced in rabbits by the nasopharyngeal secretions from cases of uncomplicated influenza. We now propose to define more exactly the nature of the peculiar or active substance responsible for these effects and to distinguish it from bacteria of the ordinary species.

Before presenting what are regarded as the decisive experiments, all of which were made in rabbits and guinea pigs, we desire to put briefly on record series of tests carried out on monkeys, *Macacus rhesus*, chiefly, in which the nasopharyngeal secretions from cases of uncomplicated influenza, collected from 12 to 48 hours after the onset of the symptoms, were filtered through Berkefeld V or N candles and injected intratracheally or subconjunctivally, or by both routes, into these animals.[2] In some instances the material was injected as it came from the filter. Occasionally it was concentrated at low temperature *in vacuo* according to the method of Amoss and Taylor.[3]

All these experiments resulted negatively in that no effects were observed which were not also obtained from similarly treated secretions from persons believed not to have suffered from influenza. Hence the work of Nicolle and Lebailly,[4] Gibson, Bowman, and

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[1] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.

[2] All operations were performed under light ether anesthesia.

[3] Amoss, H. L., and Taylor, E., *J. Exp. Med.*, 1917, xxv, 507.

[4] Nicolle, C., and Lebailly, C., *Compt. rend. Acad.*, 1918, clxvii, 607.

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Connor,[5] and Bradford, Bashford, and Wilson[6] was not confirmed by these experiments. But since in many of the monkeys employed any effect produced by the intratracheal injections might have been masked by the presence of lesions of pulmonary tuberculosis, these experiments are not regarded as conclusive. In the meantime the work with rabbits was proceeding in a promising manner and hence the latter animal was chosen for the new series of experiments.

These experiments extended our observations as follows: (1) A condition similar to that found in rabbits injected with the unfiltered nasopharyngeal secretions was obtained by employing filtrates of the lung tissue of such affected animals. (2) Filtrates of the nasopharyngeal washings from early cases of epidemic influenza also. induced similar effects in rabbits. (3) When guinea pigs were used instead of rabbits, they showed clinical and pathological effects indistinguishable from those already observed. (4) The peculiar substance inducing these effects, when submitted to the action of 50 per cent glycerol, maintained its activity without alteration in its effects.

Filtered Lung Tissue from Affected Rabbits.

It was thought advisable to employ at first, for filtration experiments, material possibly more active than the patient's nasopharyngeal secretions. The active substance was therefore carried through several rabbit passages without filtration in the following manner. The nasopharyngeal washings from Case 17, described in the first communication,[1] had been collected 12 hours after the onset of uncomplicated influenza and injected intratracheally in rabbits. As judged by the occurrence of typical effects on the blood and on the lungs, the active material was then transmitted through two successive rabbits by means of the unfiltered lung tissue of each previous animal. The lung tissue of the last rabbit, obtained at the height of the reaction, was ground with sand in sterile saline solution and filtered.

[5] Gibson, H. G., Bowman, F. B., and Connor, J. I., *Brit. Med. J.*, 1919, i, 331.

[6] Bradford, J. R., Bashford, E. F., and Wilson, J. A., *Brit. Med. J.*, 1919, i, 127.

Protocol 1.—The lung tissue of the rabbit corresponding to the third passage of this series was ground with sterile sand in saline solution[1] and the suspension centrifuged at low speed. The supernatant fluid was removed and filtered through a tested Berkefeld candle, size N. 0.5 cc. of the filtrate gave no growth on blood agar plates..

Apr. 17, 1919. 3.5 cc. of the filtrate were introduced intratracheally in a rabbit[7] whose total leucocytes were 17,500, of which 9,625 were mononuclears. Apr. 18. Total leucocytes 10,360, of which 3,936 were mononuclears. The animal had lost 100 gm. in weight; the temperature was unchanged (39.1°C.); conjunctivitis appeared. Apr. 19. Leucocyte count unchanged; temperature 40.1°C. Killed. All the organs except the lungs were normal in appearance. The lungs were voluminous, edematous, and emphysematous, and in the lower lobes diffuse hemorrhages were noted, in the upper lobes small discrete hemorrhages, especially underneath the pleura, which was apparently unaffected. On section, a blood-stained frothy fluid escaped and the hemorrhages were observed to occupy the depth of the tissues. The trachea, especially in the lower third, showed congestion and small hemorrhages and was covered with mucus.

The microscopic examination (Figs. 1 and 2) confirmed the gross appearance. There were generalized extravasations of erythrocytes into the interalveolar structures and intraalveolar spaces, localized small hemorrhages, extensive edema, and emphysema. In addition, a small amount of cellular exudate was found in the parenchyma, consisting mainly of polymorphonuclear acidophilic and of mononuclear cells, among which were larger cells of the respiratory epithelial type. The bronchi showed exfoliated epithelium, some cells of which were necrotic, and contained a mixture of red cells, coagulated serum, leucocytes, and fibrin in small amount. No growth was obtained on aerobic cultivation of the lung tissue.[8]

This experiment, which is typical of many, shows that the lesions of the lungs and of the circulating blood described in the previous paper as arising in the rabbit from the injection of unfiltered nasopharyngeal secretions from early cases of influenza can be produced by the injection of the filtered extract of the lungs of affected rabbits. The next step was the passage from rabbit to rabbit of the active material contained in the lung tissue by means of successively filtered materials.

Protocol 2.—A suspension of the lung of the previous rabbit was filtered through a Berkefeld candle, size V. The filtrate was proved sterile by aerobic cultivation tests and was introduced intratracheally into another series of rabbits. While

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[7] Several rabbits were inoculated, but only the rabbit used for further transmission experiments is described in this paper.

[8] The results of anaerobic cultivation will be reported in a later paper.

tests were made with the filtrate an alternate test with unfiltered lung suspension was carried out.

This consecutive series consisted of ten rabbits, which developed in 24 to 48 hours temperature reaction, conjunctivitis, and leucopenia, involving especially the mononuclear cells. At autopsy the animals exhibited the pathological condition of the lungs described as typical of the action of the influenzal material (Fig. 3). There was no essential difference noted in the action of the filtered as compared with the unfiltered suspensions. Aerobic cultures of the lung tissue in blood and plain dextrose broth were systematically made. As a rule no growth was obtained. But in three instances common bacterial species were found. Thus the fifth passage, in which an unfiltered suspension was used, yielded an indefinite pneumococcus; the seventh and eighth passages, in which filtered suspensions were employed, B. pyocyaneus;[9] and the tenth passage made with an unfiltered suspension, an unclassified, small, Gram-negative bacillus of hemoglobinophilic nature. The variety of bacteria appearing in these cultures indicates that they were of accidental occurrence.[10]

The series of experiments suggests that the particular clinical reactions and pathological effects induced in rabbits by the nasopharyngeal washings from early cases of epidemic influenza are due to an active substance which passes through Berkefeld filters and survives successive animal passages of the filtrate.

The preceding experiments were made with material obtained in 1919 and passed through rabbits before filtration. The recurrence of the epidemic of influenza in 1920 afforded another opportunity for the study of material from early cases of the disease in the manner indicated, and for the direct injection into the rabbit of the filtered nasopharyngeal secretions of man.

Filtered Nasopharyngeal Secretions.

Nasopharyngeal washings, obtained from two patients (Nos. 24 and 26, described in the first paper[1]) who had been ill 36 and 30 hours, were shaken in saline solution and filtered through Berkefeld N candles. The filtrates tested by aerobic culture were sterile. They were inoculated intratracheally into rabbits as follows:

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[9] Subsequent tests proved that the Berkefeld candle used in these two passages was contaminated with this organism.

[10] The part played by these aerobic microorganisms will be described in another communication.

Protocol 3.—Jan. 21, 1920. 4 cc. of the filtered nasopharyngeal washings from Patient 24 were introduced intratracheally in a rabbit whose total leucocytes were 10,400, of which 6,760 were mononuclears. Temperature, before injection, 39.2°C. On Jan. 23, after 48 hours, conjunctivitis and a temperature rise to 39.4°C. were noted. The total leucocytes fell to 6,800, of which 4,080 were mononuclears. Jan. 24. Temperature 39.6°C.; total leucocytes 8,200, of which 2,970 were mononuclears. Killed. All the organs except the lungs were normal in appearance. The right lung was more extensively involved than the left. They were both voluminous as a result of edema and emphysema. Small discrete hemorrhages were seen only in the right lung, especially under the pleura. On section the hemorrhages were noted to occupy the depths of the tissues. The bronchi contained mucopurulent material. The microscopic appearance confirmed the gross condition. There were localized small hemorrhages, edema, and emphysema. The capillaries were filled with blood. The small amount of cellular exudate in the parenchyma consisted of polymorphonuclear cells, of respiratory epithelial cells, and, especially in the interalveolar strands, of mononuclear cells. The bronchi showed necrosis and exfoliation of the lining cells and contained coagulated serum, leucocytes, and fibrin. No growth was obtained on aerobic cultivation.

Jan. 24. A second rabbit was injected intratracheally with 3 cc. of the unfiltered lung tissue suspension from the preceding rabbit. Jan. 25. Total leucocytes fell from 11,400 to 9,800, and mononuclears from 5,358 to 4,116. There was conjunctivitis but no temperature rise. Jan. 26. Total leucocytes 9,400, of which 2,256 were mononuclears. Killed. The lesions present in the lungs at autopsy resembled those of the previous rabbit and were regarded as typical. Aerobic cultures showed no growth.

Protocol 4.—The typical clinical reaction and pathological effects were induced in rabbits by means of the unfiltered nasopharyngeal secretions from Patient 26. These were described in detail in the first paper.[1]

Feb. 5, 1920. 3.5 cc. of the filtered nasopharyngeal secretions from Patient 26, collected 30 hours after the onset of symptoms, were injected intratracheally in a rabbit whose average total leucocytic count was 7,600, of which 3,331 were mononuclears. On Feb. 8, after 72 hours, total leucocytes fell to 4,050, and mononuclears to 1,539 (Text-fig. 1). Killed. The lungs showed the lesions regarded as typical. No growth was obtained in aerobic cultures.

The next passage was effected by employing the lungs of a second filtrate-injected rabbit which reacted typically, but was killed after 48 hours instead of 72. Feb. 7. Rabbit injected with 3 cc. of the unfiltered suspension of these lungs. Feb. 8. Conjunctivitis appeared and temperature rose from 39.2° to 39.4°C. Total leucocytes fell from 9,475 to 7,600, and mononuclears from 4,643 to 2,128. Feb. 9. Temperature 39.5°C.; total leucocytes 5,400, of which 1,728 were mononuclears (Text-fig. 2). Killed. Lungs showed the lesions regarded as typical. No growth obtained in aerobic cultures.
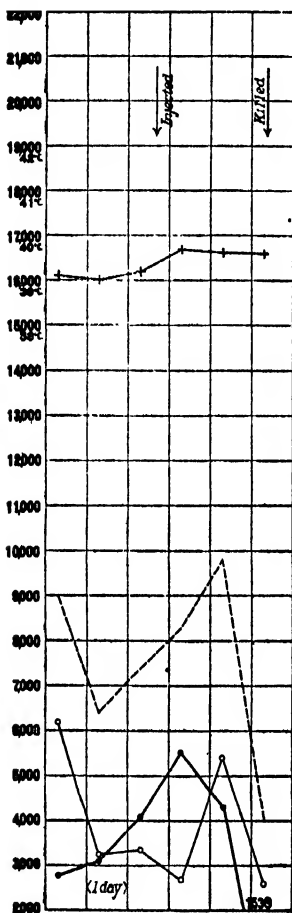
TEXT-FIG. 1.   TEXT-FIG. 2.   TEXT-FIG. 3.
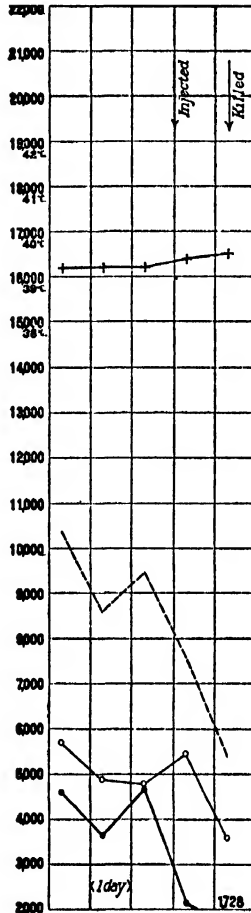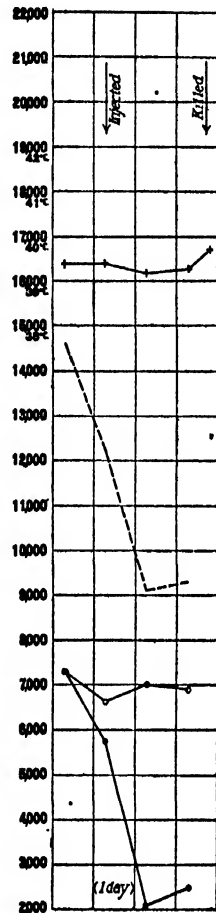
—+—Temperature
——Total leucocytic count
—•—Mononuclears
—•—Polymorphonuclears

TEXT-FIG. 1. Effect on the blood count and temperature of the intratracheal inoculation of the nasopharyngeal secretions from Patient 26. First rabbit passage. The rise in the temperature, the leucopenia, and the mononuclear depression 72 hours after inoculation are shown.

TEXT-FIG. 2. Second rabbit passage of material from Patient 26. The rise in temperature, the leucopenia, and the mononuclear depression 24 hours after inoculation are shown.

TEXT-FIG. 3. First rabbit passage of glycerolated material from Patient 6. The temperature is not correspondingly raised as shown in the other text-figures.

The lung tissue of the preceding rabbit of this series of transmissions was stored in 50 per cent sterile glycerol and after 4 months was reinjected into rabbits. The characteristic clinical reaction and pathological effects were then observed in five successive rabbit passages. In all instances no growth was obtained in aerobic cultures. These experiments will be described later.

Protocols 3 and 4 indicate that the filtered nasopharyngeal washings of early cases of epidemic influenza induce in rabbits when injected intratracheally the peculiar changes in the lungs and the blood which have been previously described[1] and are regarded as peculiar and as related to that epidemic disease in man. The first effects of the direct inoculation of the filtrates were noted in 48 hours, but after the first animal passage they were observed at the end of 24 hours. However, it should be noted that while filtrates were employed in the first inoculations the unfiltered lung suspensions were employed in the transfer from rabbit to rabbit. With material from Patient 24 the passages were carried through only two animals, while with material from Patient 26 they were carried through seven successive animals. Moreover, the glycerolated rabbit lung from this series preserved its activity for at least 4 months.

Experiments on Guinea Pigs.

The next set of experiments was carried out on guinea pigs, the material employed for inoculation being the filtered extracts of the lungs of rabbits which showed the typical lesions.

Protocol 5.—Preliminary examination of the first guinea pig gave the following results: total leucocytes 7,900, of which 2,686 were mononuclears; temperature 39.6°C.; weight 750 gm. Apr. 15, 1919, 4.30 p.m. Injected into lungs through trachea 0.65 cc. of the Berkefeld N filtrate (sterile by aerobic cultivation tests) of the suspension of rabbit lung tissue.[11] Apr. 16, 9.30 a.m. Animal sick; temperature subnormal; weight 725 gm.; total leucocytes 13,300, of which 1,862 were mononuclears. Killed. Only the distended lungs showed lesions. The latter consisted of hemorrhages, edema, and emphysema. The whole surfaces and, on section, the interior were mottled with extravasated blood. The microscopic sections showed, besides the escape of blood into the alveoli and bronchi, edema, emphysema, and a certain degree of polymorphonuclear exudation. Aerobic cultures and films showed no bacteria.

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[11] This rabbit represented the second passage of the nasopharyngeal washings from Patient 17, an influenza case described in the previous communication.[1]

Second Passage.—The lung tissue of this guinea pig was filtered through a Berkefeld V candle, and 0.5 cc. of the filtrate, sterile by aerobic cultivation tests, was introduced intratracheally into two other guinea pigs. One of the guinea pigs after 48 hours developed a leucopenia and mononuclear depression which persisted for 3 days, and was followed by a gradual return to normal. The other animal, which showed a prompt leucopenia, was killed 24 hours after inoculation. The lung lesions resembled those of the rabbits and the guinea pig of the first passage. No bacteria were obtained on aerobic culture.

The material from the lungs of this guinea pig was suspended in saline solution and injected intratracheally into other guinea pigs as shown in Table I.

TABLE I.

Results of Intratracheal Inoculation of Guinea Pigs.

Guinea pig passage.	Material inoculated intratracheally.	Effect on blood.	Remarks.	Lung lesion.
3rd	0.75 cc. of lung tissue from guinea pig of 2nd passage.	Leucopenia and mononuclear depression for 4 days.	Allowed to recover.	
3rd	0.75 cc. of lung tissue from guinea pig of 2nd passage.	Leucopenia and mononuclear depression for 2 days.	Killed.	Typical.
4th	0.75 cc. of lung tissue from guinea pig of 3rd passage.	Leucopenia, 550 mononuclears, on 3rd day after inoculation.	"	"
. 5th	0.75 cc. of lung tissue from guinea pig of 4th passage.	Leucopenia and mononuclear depression for 2 days.	Allowed to recover.	

The foregoing experiments with guinea pigs indicate that these animals respond to the intratracheal inoculation of materials derived from cases of acute influenza and passed through the lungs of rabbits very much as rabbits do. No tests were made in guinea pigs with nasopharyngeal secretions derived directly from man.

Effect of Glycerol on the Active Agent.

Since there is a class of pathogenic microorganisms (vaccinia, virus of poliomyelitis, etc.) which is resistant to glycerol, it was decided to test the action of glycerol on the active material, or agent, in the experiments described.

As a routine practice, the lung tissue derived from rabbits was cut into cubes measuring 0.5 to 1 cm., and was placed in sterile 50 per cent glycerol, which was stored in the refrigerator (4°C.). When the glycerolated tissue was to be used for inoculation it was cultured for sterility, rendered free of glycerol by washing four times in normal saline solution, and suspended as previously indicated.[1]

Protocol 6.—Total leueocytes of rabbit 12,225, of which 5,624 were mononuclears. Feb. 18, 1919. Inoculated intratracheally with 3 cc. of suspension of 5 day glycerolated rabbit lung tissue, corresponding to the ninth passage of the nasopharyngeal secretions from Case 6, an influenza patient. Feb. 19. Conjunctivitis. Leucocytes decreased to 9,125 and mononuclears to 2,099. Feb. 20. Leucopenia and mononuclear depression continued, as shown in Text-fig. 3. Killed. Lungs showed lesions regarded as typical and no growth was obtained on aerobic culture.

Feb. 20. 3 cc. of the suspension of the lung tissue from this rabbit were injected intratracheally into another rabbit whose normal total leucocytic count was 13,375, of which 6,955 were mononuclears. Feb. 21. Total leucocytes 9,750, of which 4,582 were mononuclears. A loss of 175 gm. in weight accompanied the leucopenia. This condition endured for 1 day, the animal then returning to normal.

Feb. 26. A rabbit was inoculated with 3 cc. of the suspension of rabbit lung tissue corresponding to the second passage of the nasopharyngeal secretions from another case, Patient 11.[1] In 24 hours leucopenia and mononuclear depression occurred, and persisted for 3 days; the rabbit then returned to normal.

These experiments were preliminary and show that a short immersion for 5 days in glycerol does not affect the activity of the agent. Subsequently lung tissues immersed in the 50 per cent glycerol for longer periods, 8 and 18 days, 4, 7, 9, and $10\frac{1}{2}$ months, were tested. Since there is so much similarity in the results obtained they are summarized in Table II.

From Table II it will be noted that the characteristic reaction could be obtained by the use of material immersed in 50 per cent glycerol for periods varying from 8 days to 9 months. In some instances this material was originally derived from filtrates of lung tissue. After recovery from the glycerolated lung tissue the active agent was transmitted through as many as ten successive animals. The lesions in the lungs of a rabbit inoculated with material exposed to glycerol are shown in Fig. 4. Affected lung tissue exposed to the glycerol for $10\frac{1}{2}$ months failed in two series of experiments to yield the agent in an active state.

TABLE II.

Results of Experiments with Glycerolated Lung Tissue.

Series No.	Length of exposure to 50 per cent glycerol.	No. of successive rabbits* which showed effects.	Lung lesion.	Effect on blood.	Nature of material.
	days				
1	18	10	Typical in all.	Leucopenia and mononuclear depression: 1st passage after 72 hrs.; 2nd passage after 48 hrs.; 3rd passage and subsequently after 24 to 48 hrs.	Filtered.
	mos.				
2	9	3	" " "	Leucopenia and mononuclear depression: 1st passage after 48 hrs.; 2nd passage and subsequently after 24 hrs.	Unfiltered.
3	10½	0	None.	None.	"
4	10½	0	"	"	"
5	7	3	Typical in all.	Leucopenia and mononuclear depression after 24 to 48 hrs.	Filtered.
6(Lung tissue of rabbit of 1st passage of Series 5 refiltered.)	7	5	" " "	Leucopenia and mononuclear depression within 24 hrs.	"
	days				
7	8	3	" " "	Leucopenia and mononuclear depression after 24 hrs.	Unfiltered.
	mos.				
8	4	5	" " " .	Leucopenia and mononuclear depression: 1st passage after 48 hrs.; 2nd passage and subsequently after 24 hrs.	"
9	4	3	" " "	Leucopenia and mononuclear depression within 24 hrs.	Filtered.

* The number represents, as a rule, the discontinuance of the experiment and not the cessation of the activity of the agent.

It will also be noted that the action on the circulating blood and lungs was detected usually only after 48 to 72 hours in the first passages, and, as a rule, within 24 hours in the subsequent passages.

SUMMARY.

An active transmissible agent present in the nasopharynx in early cases of influenza has been found to produce definite and characteristic clinical reactions and pathological effects in rabbits as already described in an earlier publication.[1]

The experiments here reported indicate that this active agent has the following properties.

1. The agent as it exists in the nasopharyngeal secretions in man, and in the lungs of rabbits injected with the human secretions, passes through Berkefeld V and N candles.

2. The filtered material produces the same effects on the circulating blood and on the lungs of rabbits as the unfiltered material.

3. The peculiar effects described as arising in the inoculated rabbit may also be induced in guinea pigs inoculated with the agent.

4. The agent responsible for the reaction on the blood and the lungs of rabbits withstands the action of glycerol in a sterile 50 per cent solution, for periods up to 9 months. The question must be left open at present whether the agent can withstand longer contact with the chemical. In two experiments after $10\frac{1}{2}$ months contact the agent induced no observable changes in the blood and lungs of rabbits.

EXPLANATION OF PLATES.

PLATE 32.

FIG. 1. First rabbit passage of filtered material corresponding to the fourth animal passage of the nasopharyngeal secretions from Patient 17 (Protocol 1). The extensive edema, the emphysema, the cellular exudation into the parenchyma, and localized hemorrhages are noteworthy. $\times$ 230.

PLATE 33.

FIG. 2. Section from the same rabbit as Fig. 1. This section shows particularly the number of localized small hemorrhages and the hemorrhagic extravasation into the parenchyma. $\times$ 230.

PLATE 34.

Fig. 3. The sixth passage of the nasopharyngeal secretions from Patient 17. Rabbit inoculated with filtered material (Protocol 2). The hemorrhages and the voluminous condition of the lungs resulting from edema and emphysema are noteworthy. Natural size.

Fig. 4. Rabbit inoculated with the lung tissue shown in Fig. 3 after exposure to 50 per cent glycerol for 18 days. The hemorrhages, voluminous condition of the lungs resulting from the edema and emphysema, and the absence of pneumonic consolidation are shown. Natural size.

FIG. 1.

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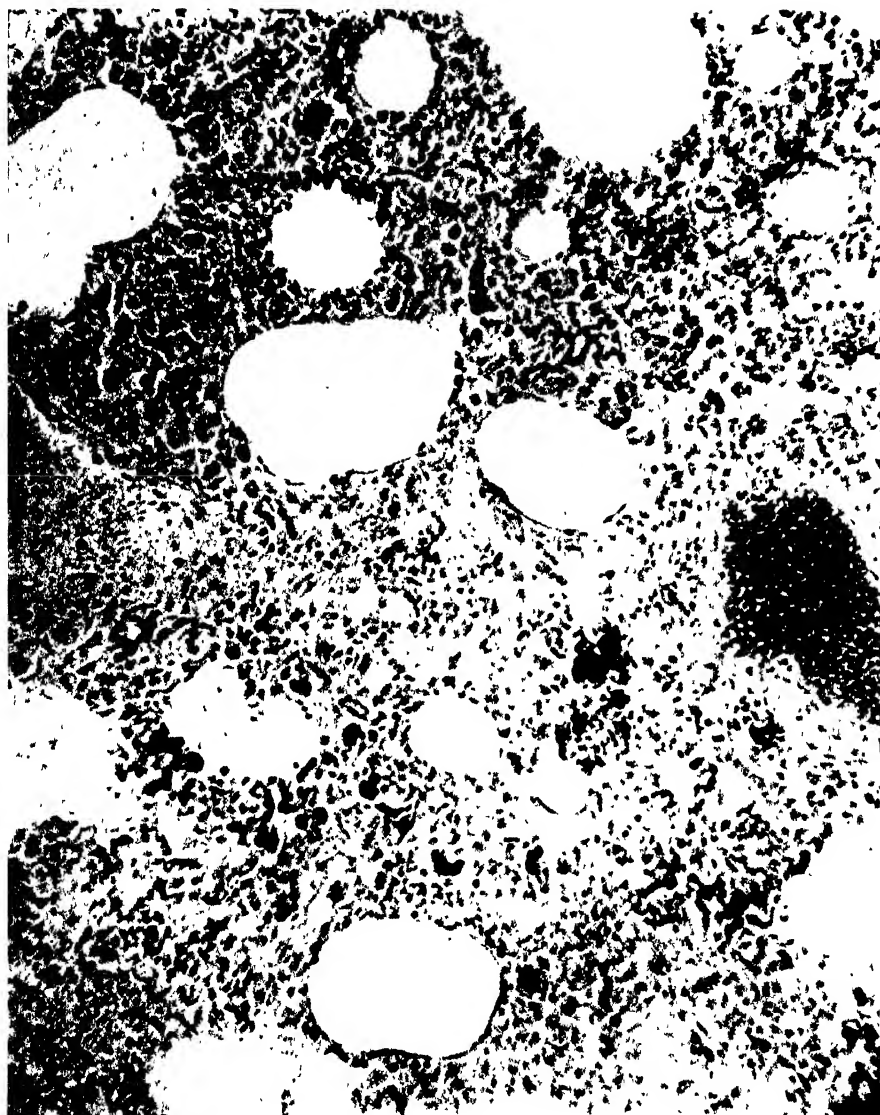
FIG. 2.

(Olitsky and Gates: Nasopharyngeal secretions from influenza.   II.)

FIG. 3.

FIG. 4.

(Olitsky and Gates Nasopharyngeal secretions from influenza

EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

III. Studies of the Concurrent Infections.

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Plate 35.

(Received for publication, December 13, 1920.)

In two previous communications [1,2] the effects produced in the lungs and blood of rabbits through the intratracheal injection of the nasopharyngeal secretions from patients in the early stages of uncomplicated epidemic influenza were described and traced to a living substance not of the nature of ordinary bacteria. Nevertheless, ordinary bacteria were occasionally, if rarely, encountered in the lungs in the course of the experiments, in which case the lesions of the lungs and the composition of the blood were quite different from those observed in the absence of ordinary bacteria. Since we had in view a possible relation of the active substance giving rise to the peculiar effects observed in the lungs and the blood to the etiological agent of influenza, and since epidemic influenza in man so commonly predisposes to a variety of secondary pulmonary infections, it was also deemed advisable to study the concurrent ordinary bacterial invasions.

The subject was approached from two points of view. First, the circumstances under which ordinary bacteria accidentally invaded the lungs in the course of the transmission experiments were considered, and second, experimental concurrent infections were induced in order to imitate in the rabbit the operation of the predisposing influences of influenza leading to secondary pulmonary affections.

As previously stated, the unfiltered[1] or filtered[2] nasopharyngeal secretions of early cases of epidemic influenza were injected into the

[1] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.

[2] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 361.

lungs of rabbits by way of the trachea.[3]   With unfiltered secretions
bacteria present in the nose and throat would, of course, be carried
into the lungs; but the bacteria often disappeared or were eliminated
in course of subsequent transmission experiments.   The kinds of
bacteria and the number of times they were observed in the various
rabbit and guinea pig passages were as follows:

Pneumococcus Type IV... 11
      "   " II atypical..............................   3
Gram-negative, hemoglobinophilic bacillus   2
B. pyocyaneus...   2
 " bronchisepticus...   2
Micrococcus catarrhalis...   1
B. coli communior...   1
Streptococcus viridans...   1
      "   hæmolyticus..   1
Gram-negative, slender, spore-bearing bacillus...................   1
Streptothrix...   1

There was no regularity in incidence or species of the microorgan-
isms found in occasional rabbit passages of a consecutive series.   For
example, in the filtrate series of ten successive passages from Case 17,[1]
the fifth passage yielded Pneumococcus Type IV, the seventh and
eighth passages *Bacillus pyocyaneus*,[4] and the tenth passage a small,
Gram-negative, hemoglobinophilic bacillus.   The filtrate series of
five such passages of glycerolated material derived from Patient 26
yielded a Gram-negative, long, slender, spore-bearing bacillus in
the fifth passage, while the glycerolated series of ten passages of
material from Case 17 yielded in the tenth passage *Bacillus coli com-
munior* and a streptothrix.   All the remaining passages in these vari-
ous series were sterile in the ordinary sense.   Obviously, therefore,
because of this variety the infections may be regarded as of accidental
nature.   In general these bacteria appeared more often in the earlier
passages of unfiltered, and in the later passages of filtered material.

That the appearance of the ordinary.bacteria was accidental is
shown by single instances of bacterial infection in several series of

[3] All operations were performed under light ether anesthesia.
[4] The occurrence of *B. pyocyaneus* was the result of faulty technique in filtra-
tion.[2]

consecutive animal passages otherwise free. Thus in the glycerolated series of Case 17 only the tenth passage yielded *Bacillus coli communior* and a streptothrix, while the filtrate in the same series showed in the fifth passage only Pneumococcus Type IV. Again the glycerolated filtrate series from Patient 26 exhibited in the fifth passage a Gram-negative, spore-bearing bacillus, and the unfiltered nasopharyngeal washings from Case 11 yielded in the third passage Pneumococcus Type IV.

In the instances in which aerobic bacteria were encountered in the first passages, the suppression of the bacteria, as already alluded to, could often be effected so that the subsequent passages were free from them. Thus in the transmissions of the nasopharyngeal secretions from Patient 6 the first passage showed Pneumococcus Type IV, while in the second, third, and fourth passages no aerobic microorganisms were found. When, therefore, the "influenzal agent," if it may be so designated for purposes of clearness, was present, its peculiar effects could be recognized in the lungs and blood. The suppression of ordinary bacteria was effected as follows: The ordinary bacteria tended to induce consolidation and even abscess of the lung and often remained localized in these areas. The influenzal agent tended to diffuse throughout the lung tissue. By killing the animals early, portions of the lungs could be selected which were free from ordinary bacteria and yet contained the active agent in a transmissible state. Of the seven patients whose nasopharyngeal secretions gave rise to typical effects in rabbits, in four the ordinary bacteria present in the washings were suppressed completely in this manner so that all the animals inoculated subsequently remained free from them.

The clinical condition of the rabbits developing concurrent infections tended to be more grave than that of rabbits in which this form of infection was avoided. The conjunctivitis was purulent instead of catarrhal. There was more loss of weight, and often the animals were prostrated and died in 36 to 48 hours.

The blood showed a greater depression of the leucocytes, involving the polymorphonuclear cells and especially the mononuclear cells in the animals which succumbed acutely; in the others an initial depression was followed in 24 to 48 hours by a leucocytosis, usually a polymorphonucleosis.

The pathological appearances also differed. Whereas in the absence of ordinary bacteria the lungs showed hemorrhagic edema and emphysema, without consolidation and pleuritis, in the presence of these bacteria a pneumonic consolidation involving one or more lobes and sometimes actual abscess with softening of the tissues arose. The several kinds of bacteria mentioned were found more or less abundantly in the consolidated areas.

It is obvious, therefore, that the effects of materials derived from cases of influenza in man on the lungs and the blood of rabbits are sharply distinguished according as common kinds of bacteria do or do not multiply in the lungs. Under the former circumstances the ordinary and usually severe inflammatory reaction follows; under the latter conditions a peculiar hemorrhagic edema of patchy character arises in the lungs, and the blood shows a marked and characteristic leucopenia with mononuclear depression.

Experimental Concurrent Infection.

It has been commonly and widely noted that cases of influenza in man, uncomplicated by concurrent infections of the bronchi and lungs, tend quickly to recover, but that when these structures are involved in ordinary bacterial infections several kinds of bacteria appear, among which are streptococci, Pfeiffer bacilli, pneumococci, staphylococci, and, rarely, meningococci and even other bacteria; in this event the disease process is correspondingly rendered more intense. The impression is widespread that the inciting agent of influenza, whatever it may be, renders the lung structures more vulnerable to these bacteria, many of which are ordinarily present in the nasopharynx in health. Hence the question at once arose whether the influenzal agent under consideration also predisposed the pulmonary structures to such concurrent or secondary infections. A series of experiments was devised to test this point. The first step was to determine the effects of pure cultures of the several bacteria alone.

Intratracheal Inoculation of Bacteria.

The microorganisms of the ordinary kind to which attention was directed were a Type IV and an atypical Type II pneumococcus, *Bacillus pfeifferi*, and *Bacillus bronchisepticus*, the last because it is a common inhabitant of the upper air passages of the rabbit.

Pneumococcus.—The strains of pneumococci employed were freshly isolated from the lungs of rabbits used in the transmission experiments, or directly from the nasopharyngeal washings and sputum from the secondary pneumonias of cases of influenza, or from the normal sputum of man.

If small numbers of the pneumococci were inoculated intratracheally in rabbits, the blood picture remained unchanged for 2 to 4 days, after which a more or less pronounced polymorphonuclear leucocytosis arose which lasted for 2 to 4 days, when the blood returned to normal. Larger doses of the pneumococci led to a prompt polymorphonucleosis, a bacterial invasion of the blood stream, and death. The lesions present in the lungs and pleura consisted of consolidation and exudate similar to that described by Lamar and Meltzer[5] and Wollstein and Meltzer.[6] Filtrates from these consolidated lung tissues were without pronounced effect on normal rabbits when injected intratracheally.

B. pfeifferi.—The *B. pfeifferi* used for inoculation was obtained from lungs of fatal cases of influenza, from exploratory thoracentesis, and from the sputum of cases of influenza. *B. pfeifferi* were suspended in saline solution (12 to 60 billion per inoculation) and injected intratracheally. Three separate strains were injected. The passage of one of the strains through two rabbits in succession and of the other two through three rabbits was attempted. The secondary passages were made with ground and suspended lung tissues of the rabbits previously inoculated. The effect of the injections was to induce a leucocytosis which was generally of a polymorphonuclear type and was still present at the end of 48 hours when the animals were killed in order to observe the lung lesions (Text-figs. 1 to 3). Two of the strains induced no visible lung lesions; the third strain seemed to set up a pneumonic consolidation from which, however, not *B. pfeifferi* but an atypical Pneumococcus Type II was isolated. In no instance was *B. pfeifferi* recovered from the injected lungs.

The toxic extract described by Parker[7] was injected into the lungs of seven rabbits in doses of from 2 to 3 cc. In certain rabbits a polymorphonucleosis was set up, in others no blood changes were produced (Text-fig. 4). No visible pulmonary lesions resulted.

[5] Lamar, R. V., and Meltzer, S. J., *J. Exp. Med.*, 1912, xv, 133.

[6] Wollstein, M., and Meltzer, S. J., *J. Exp. Med.*, 1913, xvii, 353.

[7] Parker, J. T., *J. Immunol.*, 1919, iv, 331.
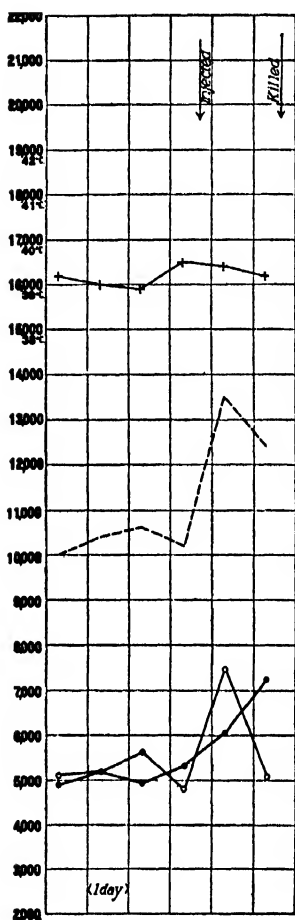
TEXT-FIG. 1.   TEXT-FIG. 2.   TEXT-FIG. 3.
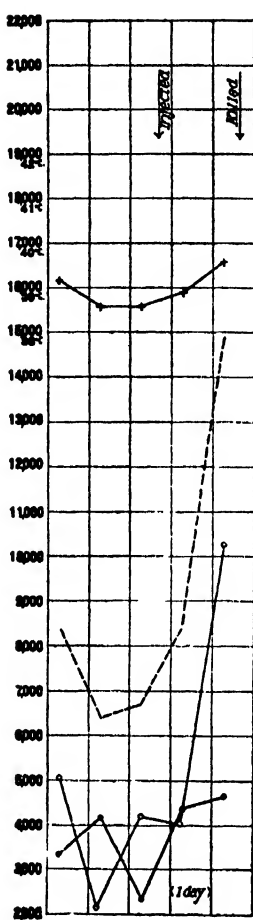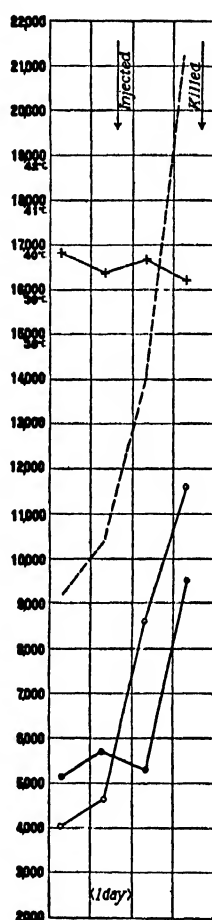
+ Temperature
— Total leucocytic count
○ Mononuclears
● Polymorphonuclears

TEXT-FIG. 1.   Effect on the blood count and temperature of the intratracheal inoculation of *B. pfeifferi*.   First rabbit passage.   The leucocytic rise, the polymorphonucleosis, and the variable temperature reaction are noteworthy.

TEXT-FIG. 2.   Effect on the blood count and temperature of the intratracheal inoculation of *B. pfeifferi*.   Second rabbit passage.   The leucocytic rise, the polymorphonucleosis, and the variable temperature reaction are noteworthy

TEXT-FIG. 3.   Effect on the blood count and temperature of the intratracheal inoculation of *B. pfeifferi*.   Third rabbit passage.   The leucocytic rise, the polymorphonucleosis, and the variable temperature reaction are noteworthy.

In other words, neither *B. pfeifferi* nor its toxic constituents when injected intratracheally into the lungs of rabbits induced lung or blood changes similar to those described for the influenzal agent, or set up a pneumonic consolidation, or led directly to the death of the animals. As a rule, a polymorphonuclear leucocytosis without distinctive lung lesions was produced.

B. bronchisepticus.—Small doses (one-fiftieth of the 18 hour growth on a standard agar slant) induced after several days a slowly developing polymorphonucleosis and leucocytosis with multiple abscesses and necrosis in the lungs; large doses (ten times as much) caused acute death.

Experimental Reproduction of Concurrent Infections

We now turned to the experiments on concurrent infection with the influenzal agent and ordinary bacteria. To review the main points reached or at issue we may state that pneumococci, *Bacillus pfeifferi* or its poison, and *Bacillus bronchisepticus*, in small doses, produce only transient effects when injected intratracheally in rabbits, and these effects differ essentially from the equally transient effects induced by the influenzal agent. It was next proposed to ascertain whether lungs already damaged by the influenzal agent would react differently to the bacterial inoculations. The method employed was to introduce the influenzal agent into the lungs and then to inject the bacteria either into the lungs or into the circulation. The latter route was adopted as a severer trial of the concomitant action, even though it may not be the route taken in the ordinary secondary infections of the lungs in man.

Influenzal Agent Plus Pneumococci.—May 31, 1919. A rabbit was injected intratracheally with 2.5 cc. of a suspension of glycerolated lung tissue corresponding to the fourteenth rabbit passage of the nasopharyngeal secretions from Patient 17, a case of influenza. June 1. Conjunctivitis; loss of weight (40 gm.); leucopenia (decrease of 2,075 cells); mononuclear depression (decrease of 612 cells). June 2. Injected intravenously with 5 million Pneumococcus Type IV, rabbit strain. June 3. Temperature 39.5°C.; total leucocytes increased 6,350 cells; mononuclears increased 4,904 cells. June 4. Temperature 40.5°C.; total leucocytes increased 2,875; mononuclears increased 54 cells. Died. The lungs showed consolidation of the right lower lobe, and hemorrhage, edema, and emphysema of other parts. From the lung and spleen Pneumococcus Type IV was isolated in culture.
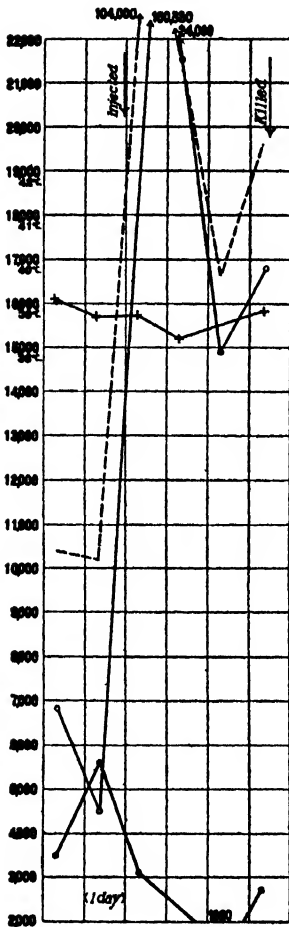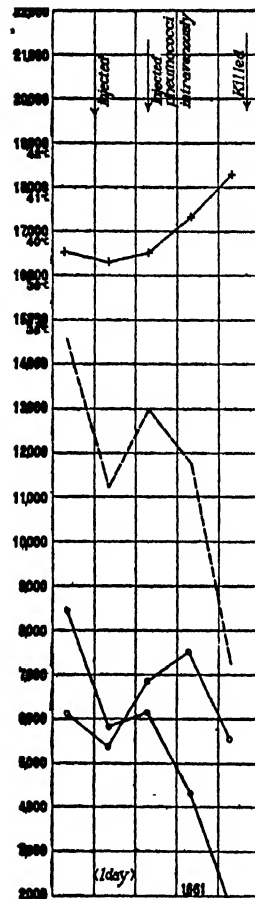
TEXT-FIG. 4.   TEXT-FIG. 5.

TEXT-FIG. 4. Inoculation intratracheally of the poison of B. *pfeifferi* as pre-pared by Parker's method. The marked polymorphonucleosis is shown.

TEXT-FIG. 5. Inoculation of a rabbit intratracheally with the influenzal agent followed in 24 hours by an intravenous injection of pneumococci. The depression in the leucocytic as well as in the mononuclear counts, and the high temperature are noteworthy. This animal was moribund when killed, and showed lobar consolidation, which yielded pneumococci, besides the typical lesions of the influenzal agent.

A second rabbit (Text-fig. 5) gave an identical result.

In order to control this experiment a third rabbit was injected intratracheally with 3 cc. of a suspension of normal rabbit lung tissue and then injected intravenously in the same manner as the preceding rabbit with 5 million pneumococci. Except for a transient leucocytosis 24 hours after the first injection, no effects were noted. The animal was killed 2 days after the injection of pneumococci. The lungs and other organs appeared normal.[8]

Influenzal Agent Plus Streptococcus viridans or B. pfeifferi.—Experiments modelled precisely on those described for the pneumococcus were carried out with *B. pfeifferi* and *Streptococcus viridans*. In each instance 24 hours after the intratracheal injection of the influenzal agent, a standard agar slant growth of *Streptococcus viridans* or one-half of such a growth of *B. pfeifferi* suspended in saline solution was injected intravenously. Preceding the intravenous injections the characteristic blood changes due to the agent were present. The animals injected with the streptococcus usually succumbed on the 3rd day and showed a massive consolidation of the lungs with fibrinous pleuritis. Cultures from the lung tissue yielded an abundant growth of *Streptococcus viridans*. A control rabbit given normal lung tissue suspension intratracheally and the streptococci intravenously was killed after 3 days, and at autopsy failed to show lung changes. No growth of the streptococcus was obtained from the lung tissue.[9]

The combined influenzal agent and *B. pfeifferi* injections set up a bronchopneumonic consolidation of one or more pulmonary lobes, and a hemorrhagic edema of the remainder of the lung. The cultures yielded abundant *B. pfeifferi*. A control rabbit injected intratracheally with normal lung suspension and intravenously with *B. pfeifferi* showed a transient polymorphonuclear leucocytosis. The lungs were not visibly altered.

These severe tests showed the conditions under which concurrent infections arose experimentally in the rabbit. A method was next employed in rabbits which imitated more closely the manner of development of secondary infections in influenza in man.

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[8] Larger or fatal doses of virulent pneumococci injected intravenously resulted in a generalized pneumococcic septicemia in rabbits inoculated intratracheally with normal rabbit tissue; all organs, including the lungs, were congested and yielded the pneumococcus on culture. The rabbits inoculated intratracheally with the influenzal agent, however, in similar procedures showed definite localization of the bacterial infection in the lungs—fibrinous and exudative pleuritis, and consolidation—besides the involvement of other organs.

[9] In the case of *Streptococcus viridans* an experiment was also made in which these bacteria were injected intravenously 4 hours before the intratracheal inoculation of the influenzal agent. The results were similar.

Influenzal Agent Injected Intratracheally with Pneumococcus Type IV, Streptococcus viridans, or B. pfeifferi.—A series of rabbits was injected intratracheally with the influenzal agent together with small non-pathogenic doses of Pneumococcus Type IV, *Streptococcus viridans*, or *B. pfeifferi.* These experiments, properly controlled, showed that effects were produced similar to those obtained when these bacteria were injected intravenously; namely, more or less extensive consolidation, either lobar or bronchial, and from the affected lungs the same kind of microorganisms which were inoculated were recovered in pure culture, while the remainder of the lungs showed the hemorrhagic edema with emphysema characteristic of the influenzal agent (Figs. 1 and 2).

The experiments just given have an important bearing on the subject of this study since they show that the intratracheal injection of the influenzal agent in rabbits exerts an influence on the pulmonary structures of these animals of a nature to encourage the invasion of the lung and the subsequent multiplication there, with lethal outcome, of such bacteria as the pneumococcus, streptococcus, and *Bacillus pfeifferi*, which otherwise, in the doses employed, are without marked effect. The control experiments show that the injection of normal rabbit lung exerts no such predisposing influence. While the experiments are perhaps not an exact reproduction of the conditions occurring in man in secondary pneumonia following influenza they bear directly on these conditions.

CONCLUSIONS.

1. Concurrent infections in the experiments described may be regarded as of accidental nature and are not causally related to the typical effects induced in rabbits by a material wholly free from ordinary bacteria.

2. The influenzal agent exerts an effect on the pulmonary tissue which encourages the invasion of the lung and subsequent multiplication there of ordinary bacteria, such as the pneumococcus, streptococcus, and *Bacillus pfeifferi*.

3. A similarity is believed to exist between the conditions under which concurrent infections arose in the inoculated rabbits and those which seem to favor the occurrence of concurrent infections during epidemic influenza in man. In no instance did death occur in the rabbits as a result of the uncomplicated effects of the influenzal

agent alone. When death occurred in any of the inoculated animals concurrent infection of the lungs by ordinary bacteria was present. The microorganisms most commonly met with under these conditions were Pneumococcus Type IV and atypical Type II, streptococci, and hemoglobinophilic bacilli. Other kinds were encountered less often.

EXPLANATION OF PLATE 35.

FIG. 1. The influenzal agent with small numbers of Pneumococcus Type IV was inoculated intratracheally in a rabbit. The right lung shows the typical effects of the influenzal agent; it is voluminous, emphysematous, edematous, and hemorrhagic. The left lung shows massive lobar consolidation (red hepatization) and yielded on culture a profuse growth of the pneumococci. Natural size.

FIG. 2. The influenzal agent with *B. pfeifferi*, both ordinarily without effect by themselves, was inoculated intratracheally in a rabbit. The left lung shows the typical effects of the influenzal agent; it is voluminous, emphysematous, edematous, and hemorrhagic. The right lung shows patchy consolidation (bronchopneumonia) and yielded on culture *B. pfeifferi*. Natural size.

Fig. 1.

Fig. 2.

IMMUNOLOGICAL DISTINCTIONS OF ENCEPHALITIS AND POLIOMYELITIS.

By HAROLD L. AMOSS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

In the course of the many discussions of encephalitis lethargica which have followed the pandemic of that unusual disease, the question of its relation to epidemic poliomyelitis has been raised either incidentally or directly. Von Economo,[1] who reported the first Austrian cases, believed that poliomyelitis could be excluded, while Draper,[2] who studied the residual pareses in some of the cases in the English outbreak of 1918, concluded that many of them were true cases of poliomyelitis; however, he called attention to certain phenomena distinctly unusual in experiences with poliomyelitis and left open the question as to whether in the whole group there existed a subsection representing a new disease. Crookshank,[3] in discussing the epidemiology, has expressed the view that poliomyelitis, lethargic encephalitis, and possibly still other paralytic epidemic affections, may be different manifestations of one etiologically simple malady.

There are grave objections to the confusion of the etiology of poliomyelitis and lethargic encephalitis. The divergent clinical manifestations have been more marked as the epidemic outbreak of lethargic encephalitis has endured and become more widely distributed. The great difference in the communicability of this affection as compared with poliomyelitis is another point of capital distinction. Apparently the seasonal variation in the prevalence, namely the occurrence of poliomyelitis in the summer and autumn months and

[1] von Economo, C., *Wien. klin. Woch.*, 1917, xxx, 581.

[2] Draper, G., *Rep. Local Gov. Bd. Pub. Health and Med. Subjects, New Series, No. 121*, 1918, 62.

[3] Crookshank, F. G., *Boston Med. and Surg. J.*, 1920, clxxxii, 34.

of lethargic encephalitis in the winter and spring months is no longer so sharp. Netter[4] has reported summer cases of the latter malady in Paris. Moreover, from information furnished by the Department of Health of the City of New York and the statistics in the *Public Health Reports of the United States Public Health Service* it appears that a similar extension of the period of prevalence is occurring in the United States.

There is one means of distinguishing epidemic poliomyelitis and lethargic encephalitis which has not yet been applied. It relates to the point whether the serum of convalescent cases of lethargic encephalitis can neutralize the virus of poliomyelitis. This fact is readily determined experimentally by the method described by Amoss and Eberson.[5] The principle of the test lies in the power of a neutralizing serum, when administered intraspinally, to prevent the development of poliomyelitis in the monkey following the intravenous injection of a large dose of the virus.

EXPERIMENTAL.

The blood serum of four cases of lethargic encephalitis was used in the test, one from a patient convalescent in the 5th week of the disease, the second in the 4th month, the third in the 5th month, and the fourth 15 months after the attack. The tests were controlled by two experiments in which the same procedure was followed, except that one monkey received intraspinal injections of normal human serum and the other intraspinal injections of poliomyelitic serum from a monkey which had had experimental poliomyelitis 9 months before and recovered with residual paralyses.

The virus of poliomyelitis used came from a strain which had been passed from monkey to monkey many times during the past 9 years and which, between passages, had been preserved in 50 per cent glycerol in the ice box. Before starting the tests this virus was passed through three normal monkeys in order to determine its virulence. The certain infecting dose for intracerebral injection[6]

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[4] Netter, A., *Bull. et mem. Soc. méd. hôp. Paris*, 1920, xliv, series 3, 1030.

[5] Amoss, H. L., and Eberson, F., *J. Exp. Med.*, 1918, xxvii, 309.

[6] All intracerebral inoculations were made under ether anesthesia.

was found to be 0.25 cc. of a Berkefeld filtrate of a 5 per cent suspension of the nervous tissues containing the virus.

The infecting power of the virus when given intravenously is shown in the following preliminary experiment.

A monkey received at 5 p.m. an intraspinal injection of 2 cc. of normal horse serum. The following morning 50 cc. of a 5 per cent suspension of fresh virus were given intravenously. 5 days later the animal was weak in both legs and excited. Both arms were paralyzed on the 6th day and the monkey died on the 7th day.

Autopsy.—Microscopic lesions of experimental poliomyelitis were found.

In making suspensions for intravenous injections the tissues used must be fresh. Accordingly, a monkey prostrate from an intracerebral injection of 0.5 cc. of a suspension of the virus 6 days before, was killed with ether and autopsied at once. A 5 per cent suspension of the cord and medulla was prepared for immediate injection.

Series 1.

Experiment 1.—Macacus rhesus A. Normal human serum control. Mar. 11, 1920, 5.50 p.m. Injected intraspinally 2 cc. of fresh normal human serum. Mar. 12, 2.30 p.m. Injected intravenously 50 cc. of a 5 per cent suspension of fresh poliomyelitis nervous tissue. 2.50 p.m. Intraspinal injection of 2 cc. of fresh normal human serum. The intraspinal injection of 2 cc. of normal human serum was repeated daily for 3 days. Mar. 18. Excited; slight head tremor and left facial paralysis. Mar. 19. Prostrate. Mar. 20. Etherized when moribund.

Autopsy.—Microscopic lesions of experimental poliomyelitis.

Experiment 2.—Macacus rhesus B. Immune poliomyelitic serum. Mar. 11, 1920, 6 p.m. Injected intraspinally 2 cc. of serum from a monkey which had had experimental poliomyelitis 9 months before and had recovered with residual paralyses. Mar. 12, 3 p.m. Injected intravenously 50 cc. of virus suspension. 3.25 p.m. Intraspinal injection of 2 cc. of poliomyelitic immune monkey serum. The intraspinal injections of 2 cc. of immune monkey serum were repeated daily for 3 days. The monkey remained well.

Experiment 3.—Macacus rhesus C. Serum from convalescent case of lethargic encephalitis. Mar. 11, 1920, 6.05 p.m. Injected intraspinally 2 cc. of serum from Case 1, age 31 years, who was in the 5th week of well defined lethargic encephalitis with general disturbance of the functions of the central nervous system and involvement of third and seventh cranial nerves. Mar. 12, 3.30 p.m. Intravenous injection of 50 cc. of virus suspension. 3.55 p.m. Intraspinal injection

of 2 cc. of encephalitis serum from Case 1. The intraspinal injection of the encephalitic serum was repeated daily for 3 days. Mar. 17. Animal slow and weak. Mar. 18. Found dead at 9 a.m.

Autopsy.—Microscopic lesions of experimental poliomyelitis.

Experiment 4.—Macacus rhesus D. Serum from convalescent case of lethargic encephalitis. Mar. 11, 1920, 6.10 p.m. Injected intraspinally 2 cc. of serum from Case 2, age 34 years, 3 months after definite attack of lethargic encephalitis in which there was general disturbance of the function of the central nervous system, involving the third and seventh cranial nerves and spinal motor roots. Mar. 12, 4 p.m. Intravenous injection of 50 cc. of virus suspension. 5 p.m. Injected intraspinally 2 cc. of convalescent encephalitis serum from Case 2. The intraspinal injection of 2 cc. of encephalitic serum was repeated daily for 3 days. Mar. 18. Excited and ataxic. Mar. 19. Prostrate. Mar. 20. Etherized when moribund.

Autopsy.—Microscopic lesions of experimental poliomyelitis.

Series 2.

The second series of tests carried out at a different time was controlled by an experiment in which normal human serum was used for the intraspinal injections as in Experiment 1. The procedure in this series was the same as in Series 1, except that normal horse serum was used for the preparatory intraspinal injection given the day before the intravenous injection of virus. The same strain of virus employed in Series 1 was again tested for infecting power and used in this series.

Experiment 5.—Macacus rhesus E. Normal human serum control. May 18, 1920, 4.10 p.m. Injected intraspinally 2 cc. of normal horse serum. May 19, 12 m. Intravenous injection of 50 cc. of virus suspension. 12.25 p.m. Injected intraspinally 2 cc. of normal human serum. The intraspinal injection of normal human serum was repeated daily for 3 days. May 23. Monkey excited; both legs weak. May 24. Complete paralysis of both legs and right facial paralysis. May 25. Died.

Autopsy.—Microscopic lesions of experimental poliomyelitis.

Experiment 6.—Macacus rhesus F. Serum from case of lethargic encephalitis. May 18, 1920, 4 p.m. Injected intraspinally 2 cc. of normal horse serum. May 19, 11.30 a.m. Intravenous injection of 50 cc. of virus suspension. 11.55 a.m. Intraspinal injection of 2 cc. of serum from Case 3, age 28 years, taken 4½ months after acute onset of lethargic encephalitis. The patient's illness began with dizziness, disturbance of vision, vomiting and fever, and he gradually became stuporous. Later paralyses referable to the seventh and spinal nerves appeared. The intraspinal injection into the monkey of the convalescent serum was repeated

daily for 3 days. May 26. Monkey had paralysis of both arms and shoulder muscles. May 27. Died.

Autopsy.—Microscopic lesions of experimental poliomyelitis.

Experiment 7.—*Macacus rhesus* G. Serum from case of lethargic encephalitis. May 18, 1920, 4.20 p.m. Injected intraspinally 2 cc. of normal horse serum. May 19, 11 a.m. Intravenous injection of 50 cc. of virus suspension. 11.25 a.m. Injected intraspinally 2 cc. of serum from Case 4, age 24 years, who had developed lethargic encephalitis 15 months before and was still under observation with residual ptosis and partial paralysis of left side of face and left leg. The intraspinal injection into the monkey of the convalescent encephalitis serum was repeated daily for 3 days. May 24. Monkey had double ptosis. May 25. Shoulder muscles paralyzed; arms and legs very weak; head tremor; animal almost prostrate. May 26. Died.

Autopsy.—Microscopic lesions of experimental poliomyelitis.

CONCLUSIONS.

Lethargic encephalitis is an epidemic disease, the main manifestations of which relate to injury inflicted upon the central nervous system and in particular the basal ganglia of the brain.

Poliomyelitis is an epidemic disease, the main manifestations of which relate to injury inflicted upon the central nervous system and in particular the gray matter of the spinal cord and medulla oblongata.

At the outset of the epidemic of lethargic encephalitis the two diseases tend to prevail at distinct and different seasons of the year, although recently cases of epidemic encephalitis have arisen in the midsummer months. The two maladies therefore are perhaps less distinguished by seasonal prevalence than has been supposed.

They are, however, distinguished by great differences in communicability to monkeys. Epidemic poliomyelitis is readily transmitted through inoculation of the affected central nervous tissues of man to monkeys, while it may still be regarded as doubtful whether lethargic encephalitis has been communicated to monkeys in this manner.   .

As the experiments reported in this paper show, the two diseases can be distinguished through the power of blood serum under certain circumstances to neutralize the virus of poliomyelitis. The blood serum of convalescent cases of poliomyelitis whether in man or monkey

possesses this neutralizing power, while the blood serum of recently convalescent cases of epidemic encephalitis is devoid of it.

On the basis of the distinguishing characters described, it is regarded as desirable at the present time to hold epidemic poliomyelitis and epidemic encephalitis as integrally distinct affections. The latter also may be infectious, yet the main lesions of poliomyelitis are present in the spinal cord, and of epidemic encephalitis in the mid-brain.

THERAPEUTIC ACTION OF N-PHENYLGLYCINEAMIDE-*p*-ARSONIC ACID (TRYPARSAMIDE) UPON EXPERIMENTAL INFECTIONS OF TRYPANOSOMA RHODESIENSE.

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In a series of papers published in November, 1919 (1), we reported results which had been obtained from the treatment of various forms of experimental trypanosomiasis with *N*-phenylglycineamide-*p*-arsonic acid, or tryparsamide. The results were such as to indicate that this drug might prove of value in the treatment of the form of human trypanosomiasis which is due to infection with *Tr. gambiense.* At the time this work was done, *Tr. rhodesiense* was not obtainable for experimental study, but as the therapy of the infections caused by this organism is becoming of increasing importance, it was deemed advisable to determine the effects which might be hoped for from the use of this drug in the treatment of Rhodesian sleeping sickness, and something of the method of treatment which might be employed in these cases.

Accordingly, a strain of *Tr. rhodesiense* was obtained and a series of experiments carried out upon various forms of the animal infection including that in mice, rats, and rabbits.

Tr. rhodesiense usually exhibits a higher degree of virulence for laboratory animals than *Tr. gambiense.* The organism used in these experiments, however, was distinctly less virulent for all the animals used, except the rabbit, than the strains of *gambiense* with which we had worked. After serial passage through mice and rats, it developed a fair degree of virulence for both species of animals and was highly virulent for rabbits, but possessed very slight virulence for guinea pigs. When inoculated into mice and rats according to the procedure described in a previous paper (2), the incubation period of the infection

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was from 2 to 3 days in the case of mice and 3 to 5 days in rats.   Infected mice died in from 10 to 12 days, while rats survived a few days longer.   Rabbits were also readily infected; well marked symptomatic manifestations of disease developed in less than a week after intravenous inoculation, and the infection terminated fatally within 2 to 4 weeks.

Attempts to transmit the infection to guinea pigs were at first unsuccessful, but eventually a low grade infection with a prolonged incubation period and a low mortality was produced and serial passages were maintained through several generations of transfers.

<center>EXPERIMENTAL.</center>

The general plan of the therapeutic experiments carried out was the same as that employed in our previous work; that is, the trypanocidal action of the drug was studied by the use of the simpler blood stream infections of mice and rats, and the results thus obtained were applied to the treatment of the more complex disease as it appears in the rabbit.   Mice and rats were inoculated intraperitoneally and treated 24 hours later by the intraperitoneal administration of a single dose of the drug.   Repeated blood examinations were made to determine the effects upon the infecting organisms.   Mice were kept under observation for 1 month after treatment and rats for 2 months, as a means of determining the probable results of treatment.

Rabbits were inoculated intravenously and treated by the same route when well marked symptoms of disease had developed; the period of observation was 3 months, although most of the animals in this series were held as long as 4 months.

<center>*Therapeutic Effects.*</center>

Mice.—Several series of mice were treated with doses of from 0.15 to 1.75 gm. per kilo of body weight.   Doses below 0.25 gm. per kilo were found to exercise very little if any effect upon the general course of the infection and those of from 0.25 to 0.5 gm. merely served to delay its progress.   No cures were obtained until the dose of drug given reached 0.75 to 1 gm. per kilo.   As an example of the therapeutic effects obtained, the detailed results of one of the experiments are given in Table I.

TABLE I.

*Results from the Treatment of a 24 Hour Infection of Tr. rhodesiense in Mice.**

Dose per kilo.	No. of mice.	No. of relapses.	No. of probable cures.
gm.			
1.75	2	0	2
1.50	2	0 .	2
1.25	2	1	1
1.00	2	2	0
0.75	2	1	1

* Controls survived 11 days.

Rats.—It was found that the rat infection of *Tr. rhodesiense* was also difficult to influence with this drug. The results obtained from experiments carried out under the same conditions as with mice may be illustrated by the experiment given in Table II.

TABLE II.

Results from the Treatment of a 24 Hour Infection of Tr. rhodesiense in Rats.

Dose per kilo.	No. of rats.	No. of relapses.	No. of probable cures.
gm.			
0.75	3	0	3
0.60	3	2	1
0.50	3	1	2
0.35	3	3	0

While smaller unit doses of the drug were capable of curing the infection produced in rats than in mice, when considered from the standpoint of the ratio of the curative to the lethal dose, the figures obtained in the two instances were in very close agreement, with a slightly better ratio for the mouse. Thus it was found necessary to administer upwards of two-thirds of the maximum dose in order to effect a complete sterilization in the case of the mouse, and while cures might be obtained with only two-thirds of the maximum dose in rats, the full dose was necessary to assure such a result.

These figures were quite different from those which had been obtained with *Tr. gambiense* under similar conditions. Comparable results were obtained in mouse and rat infections of this organism

in doses as small as one-ninth and one-fifth respectively of the maximum dose for the two animal species. It appeared, therefore, that greater difficulty might be anticipated in the treatment of the more severe chronic infection of rabbits.

Rabbits.—In tests of the therapeutic effects against *rhodesiense* infection in rabbits, the animals used were inoculated intravenously with 1 cc. per kilo of body weight of a + to + + suspension of trypanosomes prepared from rat blood. This produced a very intense infection, symptoms appearing in individual animals within 3 to 5 days after inoculation, and the majority of the animals showed well marked manifestations of disease by the end of the 1st week. The controls of the series survived for only 2 weeks. Three types of experiments

TABLE III.

Results from the Treatment of Tr. rhodesiense Infections in Rabbits by the Intravenous Administration of a Single Dose of Tryparsamide Given 1 Week after Inoculation.

Dose per kilo.	No. of rabbits.	No. of relapses.	No. of probable cures.
gm.			
0.75	3	1*	2
0.60	3	2†	1

* Relapse 19 days after treatment.
† Relapse 19 and 22 days after treatment.

were undertaken in this series: (1) the use of large single doses, (2) the use of repeated smaller doses, and (3) an intensive treatment of relapses.

Single Doses.—In view of the marked resistance exhibited by *Tr. rhodesiense* in mice and rats, the single dose treatment of rabbits was placed at the highest possible level consistent with safety. A small series of rabbits was treated, with the results recorded in Table III.

Repeated Doses.—The attempt was also made to determine whether any form of repeated dose therapy based upon the use of smaller doses than those described above might prove efficacious in this class of infection. Two rabbits were given doses of 0.4 gm. per kilo at intervals of 3 days. One of these received six doses, and after a rest

period of 1 week, the treatment was resumed with nine doses at intervals of 2 to 3 days. The animal thus received a total dose of 6.0 gm. of the drug per kilo of body weight within a period of 40 days. With the second animal the number of doses was reduced to six, or a total amount of 2.4 gm. of the drug per kilo was given within 15 days. Both these animals showed rapid improvement and remained in excellent condition throughout the course of treatment. Signs of the disease disappeared completely and there was no recurrence within the period of observation. They may, therefore, be regarded as probable cures.

The same procedure was followed with three other rabbits with a still smaller unit dose of the drug—0.3 gm. per kilo. One animal received six doses of the drug at intervals of 3 days, or a total of 1.8 gm. in 15 days; the second received five doses followed by a rest period of 1 week and then a second series of ten doses, or a total of 4.5 gm. was given in 43 days; the third rabbit was given fifteen consecutive doses at regular intervals, the 1 week interim between series being omitted.

The first of these animals did well under the treatment; symptoms of disease disappeared promptly and there was no recurrence. The initial effects were much the same in the second animal, but although the course of treatment was much more prolonged, it proved ineffectual. Within 4 days after administration of the drug had been stopped, signs of the disease reappeared, and the animal died from trypanosomiasis 15 days later. With the third rabbit, which was given the same amount of drug in consecutive doses, a cure was effected.

This small group of cases furnishes an excellent illustration of the variability of individual results which may be obtained in the treatment of infections of this type and possibly also the effects which may be produced by apparently minor variations in the course of treatment.

Relapses.—In view of the great difficulty experienced in influencing *rhodesiense* infections, little was to be expected from an attempt to treat these infections once they had relapsed. It was considered worth while determining, however, whether it was possible to obtain any effect from the use of the drug in this class of cases. Three experiments of this kind were carried out.

The first case treated was a rabbit which had relapsed from a single dose of 0.75 gm. per kilo. On the 3rd day after the symptoms of the disease reappeared, treatment was begun with 0.5 gm. per kilo, and nine doses of the drug were administered at intervals of 2 to 3 days, or 4.5 gm. per kilo in 21 days. This was considered to be as intensive treatment as could be undertaken and in this instance apparently effected a cure. The animal when inoculated weighed 1,625 gm.; when retreated after relapse, 1,600 gm.; when treatment was discontinued, 1,875 gm.; when discarded, 1,900 gm. This experiment furnishes an excellent example of the remarkable tolerance exhibited by animals towards the drug and the improvement in general condition which usually follows its administration.

A second animal which had relapsed from a single dose of 0.6 gm. was given five doses of 0.5 gm. and three doses of 0.6 gm. in 18 days, but the treatment was ineffectual and relapse occurred 1 week after it was discontinued.

In a third rabbit, the effect of the treatment was uncertain. This was likewise a case of relapse from a single dose of 0.6 gm. per kilo. The animal was in poor condition when relapse occurred and retreatment was instituted. Two doses of 0.5 gm. per kilo were given with 48 hours intervening. The signs of trypanosomiasis were but slightly improved, and the animal was weaker, so the dose of drug was reduced to 0.3 gm. and two doses of this size were administered. Death occurred 3 days later without complete disappearance of the manifestations of trypanosomiasis.

By comparing these results with those previously reported from the treatment of rabbits infected with *Tr. gambiense*, it is at once apparent that the *rhodesiense* infections were much more difficult to influence. Results comparable with those obtained with single doses of 0.6 to 0.75 gm. per kilo could be obtained in *gambiense* infections with approximately one-third of this amount of drug, and while cures were uniformly obtainable in *gambiense* infections with from 0.3 to 0.35 gm. per kilo, there appeared to be no single dose of the drug with which this could be accomplished with safety in cases of *rhodesiense* infection.

CONCLUSIONS.

With the three classes of animal infections studied, the trypanocidal action of tryparsamide was found to be much less for *Tr. rhodesiense* than for *Tr. gambiense*, and a correspondingly greater difficulty was experienced in the treatment of the chronic tissue infections of *Tr. rhodesiense.*

This is, of course, entirely in accord with past experience in the treatment of human cases of trypanosomiasis due to the two organisms. It is still possible that something may be accomplished by the use of tryparsamide in cases of Rhodesian sleeping sickness on account of the tolerance exhibited to the drug and the possibility of employing an intensive system of treatment. Much less is to be expected, however, than in cases of infection due to *Tr. gambiense.*

SUMMARY.

A series of experiments was carried out to determine the effects of tryparsamide upon the infections produced in various species of animals by *Tr. rhodesiense.*

The strain of trypanosome used was one which possessed a very low virulence for guinea pigs, was fairly virulent for mice and rats, and highly virulent for rabbits.

Therapeutic experiments carried out on 24 hour infections in mice and rats showed that cures could be obtained in this class of infections by the administration of a single large dose of the drug amounting to approximately two-thirds of the maximum tolerable dose, as contrasted with similar results in cases of *gambiense* infections from approximately one-ninth and one-fifth of this dose respectively.

With advanced infections in rabbits, there appeared to be no single dose of the drug capable of insuring a cure which could be administered with safety, although some cures were obtainable with doses approximating the maximum tolerable dose. Treatment of these infections could be carried to a successful conclusion, however, by an intensive system of treatment in which large doses of the drug were administered at short intervals of time, and even relapses yielded to this treatment in some instances. The employment of such a method of treatment was possible on account of the unusual tolerance exhibited by animals to this drug, a fact which was previously emphasized.

These facts indicate that the outlook for the drug in the therapy of Rhodesian sleeping sickness is much less hopeful than in *gambiense* infections, though it is felt that some benefits may be derived from its use.

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EXPERIMENTAL STUDIES ON YELLOW FEVER IN NORTHERN PERU.

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Leptospira icteroides, first isolated from certain cases of yellow fever in Guayaquil,[1] and later from a case of this disease in Merida,[2] had assumed such significance in the study of the etiology of yellow fever as to make further investigations advisable. Peru, which has had many visitations of yellow fever, was again invaded in June, 1919, in the province of Piura, the northernmost region, bordering on Ecuador. From this invasion a small epidemic arose which had not entirely disappeared in May, 1920. During the outbreak the following towns were affected: Sechura, Morropon, Tambogrande, Chulucanas, Piura (500 cases among the 10,000 inhabitants) in 1919, and Payta (108 cases among 3,000 inhabitants) in 1920. The mortality was estimated to have been about 10 per cent, which is considerably lower than was the case with yellow fever in Guayaquil and Merida, where it was about 50 per cent. An expedition to Peru was therefore undertaken.[3] The present report deals with the results of bacteriological studies at Payta, Piura, and Morropon extending over a period of 3 months, March, April, and May, 1920.

When one of us reached Peru (March 1, 1920) Payta was the only town where the epidemic of yellow fever was still in progress; the last case occurred there on April 16. The first experiments were

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[1] Noguchi, H., *J. Exp. Med.,* 1919, xxix, 547, 565, 585; xxx, 1, 9, 13, 87, 95, 401; 1920, xxxi, 135, 159.

[2] Noguchi, H., and Kligler, I. J., *J. Exp. Med.,* 1920, xxxii, 601, 627.

carried out in Payta. In April an epidemic was reported at an inland town, Morropon, and a trip was made to that place to secure material for further studies to be carried out in Piura, where better laboratory facilities were available.

Studies in Payta.

Payta, a town of about 3,000 inhabitants, is the principal port in northern Peru. It consists of a cluster of bamboo huts on a strip of sandy shore. Rain is rare, and water is very scarce. The water supply comes from a river about 7 kilometers from the town, but the amount is hardly sufficient for ordinary daily needs.

A provisional laboratory was set up in Payta in a small bungalow consisting of three rooms. One room served as a laboratory, another as an animal room, and the third as a sleeping room. The laboratory supplies brought from The Rockefeller Institute had been resterilized in the laboratory of the Municipal Institute of Hygiene in Lima, as there were no facilities for steam or hot air sterilization at Payta. The rabbit serum used in the culture media was brought from New York and as a result of the long voyage in a tropical climate a precipitate had appeared in it. The guinea pigs, also brought from New York, had suffered severely, and about two-thirds of them had succumbed within 2 weeks of their arrival in Payta. The feed for the guinea pigs was scarce, so that only the larger and hardier animals survived. This was unfortunate, as the larger animals are less suitable for initial inoculation; however, a certain number of native guinea pigs was procured. A very serious circumstance was the fact that, owing to the lack of electric current, the dark-field microscope could not be used. Moreover, the effort to obtain Giemsa preparations of the blood were unsuccessful because of the quality of the water. Since most yellow fever patients were treated by their physicians at their own homes it was not always easy to secure consent to obtain blood for inoculation, and it was practically impossible to obtain blood twice from the same patient. Finally, the cultures of *Leptospira icteroides* brought to Payta from Merida did not survive the journey.

Under the adverse conditions and the lack of laboratory facilities, the bacteriological work was confined to cultivation and animal

transmission with such samples of blood as could be obtained, the object being to infect guinea pigs and to produce the characteristic symptoms and lesions in these animals. In all, nine cases of yellow fever were studied. Guinea pigs brought from New York were inoculated from seven and native guinea pigs from two of the cases.

Case 1 (Fatal).—B. Onset Mar. 10, 1920. Mar. 12. Temperature 39°C.; pulse 110; albuminuria. Mar. 14. Temperature 38.5°; pulse 88; albumin 1 gm. per 1,000 cc. Mar. 16. Delirious; black vomit; anuria. Mar. 17. Died.

Mar. 12 (3rd day of illness). Blood taken and cultures made (nine tubes). Mar. 16. 1 cc. of citrated blood (kept in the ice box for 4 days) was inoculated intraperitoneally into Guinea Pig 1 and 2 cc. into Guinea Pig 2. The culture tubes, which had stood at room temperature for 4 days, appeared free from contamination, and three guinea pigs were inoculated with material from Tubes 1 to 4 and three with material from Tubes 5 to 9. Some of the culture material was left standing until Mar. 23, when it was inoculated into three guinea pigs.

The two guinea pigs inoculated with blood 4 days old showed no fever. A few old hemorrhagic areas were found in the lungs of Guinea Pig 2 when it was killed on the 15th day. Of six guinea pigs inoculated with culture material 4 days old, three had fever on the 6th day and showed petechial lung hemorrhages when killed on the 15th day. The three remaining animals either suffered from an intercurrent infection (pneumonia) or escaped any obvious infection.

Of the three guinea pigs inoculated with the 11 day culture material, one (Guinea Pig 43) showed petechial hemorrhages in the lungs when killed on the 15th day. The other two showed on autopsy no leptospira lesions but enlarged spleen and pulmonary congestion, which were taken as evidence of secondary infection.

The findings described show that in no instance was a fatal infection by *Leptospira icteroides* induced, but they raise the question whether the characteristic hemorrhagic areas in the lungs in Guinea Pigs 2, 3, 5, 8, and 43 did not indicate a mild infection with this organism.

Case 2 (Moderate; Recovered).—C. Onset Mar. 13, 1920. Moderate case; course typical. Mar. 20. Recovered.

Mar. 14 (2nd day of illness). Blood taken and used wholly for making cultures. Three guinea pigs were injected with a 3 day culture, three with a 4 day culture, and three with a 9 day culture.

Of this series almost all, except Guinea Pig 14, which received a 3 day culture, and No. 19, which received a 4 day culture, died of intercurrent infections (pneumonia, paratyphoid, cocci), while two remained well. Guinea Pig 14 showed on the 5th day a temperature of 39.7°C., and on the 6th 39.6°, while at autopsy the lungs showed several hemorrhagic areas. Guinea Pig 19 showed on

the 7th day a temperature of 39.5° and on the 8th and 9th days 39.8°. It was killed on the 10th day for examination and transfers. Few small petechiæ in the lungs and minute points of hemorrhage in the right kidney. The blood and organ emulsions from this animal were inoculated on Mar. 28 into two guinea pigs, both of which soon returned to normal.

The experiments on Case 2 are suggestive and lead to the tentative conclusion that in two at least of the nine guinea pigs inoculated with culture materials prepared with blood drawn on the 2nd day a mild infection with *Leptospira icteroides* was induced. It is possible that Guinea Pig 19, if allowed to live longer, might have developed a typical form of the *icteroides* infection, as, when killed on the 10th day, definite lesions were present in the lungs and kidney. The failure to transfer the infection from this animal into two others is not conclusive, as in the early transfers a larger number of guinea pigs should be employed, because of the resistance to infection which certain guinea pigs usually exhibit.

Case 3 (recovered) gave similar results with blood drawn on the 3rd day of illness.

Two guinea pigs were inoculated with 2 cc. of blood from Case 4 (fatal; blood drawn on 2nd day of illness) soon after it was drawn, and two with the same amount of blood from Case 5 (recovered; blood drawn on 3rd day of illness). Cultures made with blood from each of these patients were left at room temperature for 4, 7, and 10 days and then inoculated into eight guinea pigs (Mar. 19, 22, and 26). Some of the animals inoculated with material from Cases 4 and 5 had definite febrile reactions and showed at autopsy lung lesions suggestive of a mild leptospira infection, but there was no fatal infection with typical jaundice.

With Case 6 (recovered; blood drawn on Mar. 19, 2nd day of disease) and Case 7 (recovered; blood drawn on Mar. 20, 3rd day of disease), the blood was drawn into citrate serum agar mixed in equal parts, and 3 cc. of the mixture were inoculated into each of two guinea pigs. Cultures made in the usual way were allowed to stand at room temperature for 3 to 7 days, and two sets of six guinea pigs were inoculated with this material. The results with Cases 6 and 7, both with blood and cultures, were unsatisfactory. The majority of the guinea pigs showed irregular febrile reactions, and from these animals, owing to the scarcity of guinea pigs, no transfers were made. Some of them, when killed later, were found to have hemorrhagic areas in the lungs, some showed indications of secondary infection (enlarged spleen), while others showed no lesions. In no instance was there a typical fatal leptospira infection.

Two more cases were studied before the epidemic in Payta subsided, Case 8 (recovered; blood drawn on 3rd day of illness, Mar. 29) and Case 9 (recovered;

blood drawn on Apr. 1, 2nd day of illness). In view of the failure to secure a definite transmission with fatal outcome with the larger guinea pigs still alive from the lot brought from New York, we decided to test native guinea pigs. For this purpose six native and two American guinea pigs, weighing 600 gm., were inoculated with a 7 day culture of the blood of Case 8 and five native guinea pigs with the 10 day culture from Case 9. None of the animals developed a typical fatal leptospira infection, although some undoubtedly had a mild infection, since lung lesions were found at autopsy and in two instances there was a suggestion of jaundice. It is interesting to note here that later experiments demonstrated that the native guinea pigs possess a greater resistance to the *icteroides* infection than the domesticated variety brought from the United States.

It is obvious that in the transmission experiments just described as having been carried out at Payta, no typical instance of fatal infection with *Leptospira icteroides* was obtained, and in no instance was the leptospira observed under the microscope. As the darkfield microscope was not available and no proper Giemsa staining could be secured, the latter circumstance is without value.

On the other hand, certain positive results were obtained in inoculated guinea pigs which led to the belief that a mild form of *Leptospira icteroides* infection had in some instances been induced; *i.e.*, rise of temperature after the period of incubation common in this infection (3 to 5 days) and at autopsy definite hemorrhagic areas in the lungs and in one instance in both lungs and kidney, with occasionally a suggestion of jaundice. The failures to obtain more pronounced results are not difficult to account for. As stated above, almost all the guinea pigs of the most favorable age and weight shipped from New York succumbed *en route* or soon after arriving at Payta. Those remaining were so few in number that they were used sparingly; hence fewer were injected with given samples of blood or cultures than would ordinarily have been employed. The rabbit serum which is essential to successful cultivation of the leptospira had undergone changes with the formation of a precipitate, and the reaction became so alkaline as to prevent a growth of *Leptospira icteroides* to any extent. And yet a degree of success, which was confirmed by subsequent results, was, we believe, achieved.

Studies in Piura.

The epidemic having subsided in Payta, the laboratory was removed to Piura, where sterilizing facilities and adequate water were available. A laboratory was set up, through the cooperation of the government, in the Belan Hospital. A detached building was used for animal quarters and feed was also more plentiful.

By the time the small laboratory had been started cases of yellow fever were reported in Morropon, a small town of 2,000 inhabitants in the foothills of the Andes. The distance from Piura was about 65 miles, a desert separating the two places. Arrangements were made at once to investigate the cases.[4] As the journey was made on horseback it was obviously out of the question to transport experimental animals, etc., by this means through a tropical region; hence it was decided to rely entirely on cultures. Fresh rabbit serum was obtained from local rabbits, and in order to guard against adverse changes in the culture media the component parts, consisting of serum, and 0.3 per cent semisolid agar, were carried separately.

The cultures were made by drawing the blood from an arm vein of the patients directly into the tube of semisolid agar, the rabbit serum then being added in a proportion of 1:5. The whole was thoroughly mixed and covered with a layer of liquid paraffin, and the tubes were carefully capped with tin-foil and carried back to Piura.

On arrival at Morropon it was ascertained that cases of yellow fever had been occurring for some time, and the epidemic was regarded as declining; however, by making house to house visits several cases diagnosed as yellow fever by Dr. Caballero were found on April 23. Between April 24 and 27 cultures with the blood were made from six cases, one of which proved later not to have been yellow fever. The remaining five cases pursued a clinical course which left no doubt as to their yellow fever nature. Because of the illness of one member of the party, the work at Morropon was suspended on April 28.

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[4] The journey on horseback from Piura to Morropon usually takes 1½ days. Our party consisted of Dr. Enrico Caballero, the government expert stationed at Catacaos, who showed us every courtesy, Mr. John Mitchell, a sanitary engineer, and Dr. Kligler. The expedition started from Piura on April 21 and arrived at its destination on April 22.

The party returned with the cultures to Piura, reaching there on May 3. At the same time the stock of guinea pigs had been renewed, 300 young, healthy animals having been brought from The Rockefeller Institute. Moreover, because of the lack of electric lighting facilities which had made it impossible to use the dark-field microscope, a storage battery suited to that instrument had also been brought from New York.

Very few of the tubes showed contamination, the blood still appearing bright red in the upper zone of the media. The cultures were inoculated into guinea pigs on May 6, or 9 to 12 days after they had been set up in Morropon. The inoculation procedure was identical with that employed in Merida[2], the upper portions of several selected tubes of culture from a case being pooled and the mixture inoculated intraperitoneally into six young normal guinea pigs.

Dark-field examination of the culture tubes undertaken the next day (May 7, 10 to 13 days after they were made) revealed the presence of active leptospiras in the cultures from three of the five cases. They were few in number and required careful search in some instances. In some tubes no leptospira was detected. As the details of the experiments show, the inoculation of cultures from four of the five cases induced typical fatal infections in some animals, other animals showing only a mild infection or escaping infection altogether.

Case 10 (Severe; Recovered).—C. M., male, age 16 years; born in Salitral; resident of Morropon. Onset Apr. 22, 1920, 7 p.m. Headache; backache; pains in muscles; nausea; no vomiting. Apr. 23. Temperature 39.6°C.; pulse 106. Apr. 24. Temperature 39.9°; pulse 100; albumin +. Apr. 25. Temperature 39°; pulse 90; albumin ++; nausea. Apr. 26. Temperature 37.4°; pulse 90; albumin ++; black vomit. Apr. 27. Temperature 37.6°; pulse 78; albumin ++; epigastric pain; epistaxis; urine increasing towards normal amount; jaundice. Apr. 28. Temperature 37°; pulse 76; recovering.

Blood was taken on the 2nd day of illness at 11 a.m. Cultures examined after 12 days contained living leptospiras. Six guinea pigs (Nos. 13 to 18) were inoculated with material from Tubes 1 and 2 on May 6, with positive transmission in all.

Guinea Pig 13.—Temperature 39.2° on the 5th day. Died on the 6th day.

Autopsy.—Epistaxis; subcutaneous petechiæ; marked hemorrhages in lungs and gastric mucosa; jaundice slight.

The emulsions of kidney and liver were inoculated into three guinea pigs (Nos. 39 (Chart 1), 40, and 41), all of which developed typical fatal infection, dying

7, 14, and 15 days after inoculation. The leptospiras were found and successful subcultures made.

Guinea Pig 14.—Killed, when moribund, to obtain infective material for therapeutic experiments to be described in the following paper.[5]

Autopsy.—Numerous petechiæ in lungs; hemorrhage and blood in stomach; liver slightly degenerated; kidneys congested; spleen normal.

Guinea Pig 15.—Killed for transfer 4 days after inoculation, at first rise of temperature to 39.4°C.

Autopsy.—No lesions were noted, but all three of the guinea pigs (Nos. 36, 37, and 38) inoculated with blood and emulsions of liver and kidneys succumbed with typical infection.

Guinea Pigs 16 and 18.—Developed characteristic infection. When jaundice appeared they were utilized for testing the curative effect of the anti-*icteroides* immune serum brought from The Rockefeller Institute, as will be described in the following paper.[5]

With this case leptospiras were found in the initial culture, with which a typical infection was induced in guinea pigs, and further transfer from animal to animal was accomplished. Pure cultures of the leptospira were in turn recovered from the infected guinea pigs.

Case 11 (Mild; Recovered).—O. V., male, age 18 years; native of Morropon. Onset in afternoon of Apr. 21, 1920. Chills; headache; backache; fever. First seen morning of Apr. 24. Temperature 40°C.; pulse 100; albumin +. Apr. 25. Temperature 38.5°; pulse 70; albumin + +; nausea, but no vomiting. Apr. 26. Temperature 38.5°; pulse 66; albumin + +. Apr. 27. Temperature 37.2°; pulse 58; albumin +; abundant urine. Apr. 29. Temperature 36.6°; pulse 50; mild jaundice; recovering.

Apr. 24 (3rd day of illness). Blood was drawn. The dark-field examination of cultures 13 days after they were made failed to reveal any leptospira, owing to accidental contamination of the tubes while they were being handled the previous day for animal inoculation.

May 6. Six guinea pigs (Nos. 25 to 30) inoculated with culture, then 12 days old. Three of these (Nos. 25, 26 (Chart 2), and 29) developed typical severe infections, while the remaining three showed no perceptible symptoms. When killed for examination, however, all showed some hemorrhagic areas in the lungs, indications of a mild infection. It is interesting to note the different results with the same culture material, due to variations in individual susceptibility of the guinea pig to *Leptospira icteroides*. The symptoms and lesions in fatally infected animals were altogether typical and hence will not be described in detail except in unusual instances. Leptospiras were found in the organ emulsions and a culture was obtained from the blood.

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[5] Noguchi, H., and Kligler, I. J., *J. Exp. Med.*, 1921, xxxiii, 253.

Transfer from one of these animals was made into three guinea pigs, all of which succumbed in due time to typical fatal infection.

One of the guinea pigs (No. 25) was used, when near collapse, for testing the efficacy of the anti-*icteroides* serum. The animal recovered, having received 1 cc. of the serum.

Case 12 (Fatal).—P. C., male, age 28 years; native of mountainous region. Onset, Apr. 23, 1920, typical. Apr. 25. Seen for the first time; temperature 39.4°C.; pulse 102; albumin ++; epigastric pain; no vomit. Apr. 26. Temperature 34.8°; patient in state of collapse; bleeding from nose and gums; black vomit; jaundice. Apr. 27, 6 a.m. Died.

Blood was taken in the morning of the 3rd day of illness. Cultures contained living leptospiras when examined on May 7 (12 days old).

May 6. Six guinea pigs (Nos. 1 to 6) were inoculated with culture material. Of these, three (Nos. 2, 3, and 4) developed severe infections, one dying on the 7th, and one on the 8th day (Chart 3), and the third being killed for transfer on the 6th day, when it was intensely icteric. Three guinea pigs inoculated with blood and liver and kidney emulsions from this animal died with typical symptoms. Three of the six original guinea pigs showed no sign of infection (Nos. 1, 5, and 6), but examination after 12 days revealed hemorrhagic areas in the lungs and also, in one instance, in the suprarenal. Leptospiras were found in the blood and organs in some of the guinea pigs, and cultures were obtained from the blood. The outstanding feature of this strain was the predominance of jaundice which it produced in the animals.

Case 13 (Moderate; Recovered).—J. C., male, age 14 years; native of Morropon. Onset Apr. 23, 1920. Apr. 26. Seen for the first time; epistaxis; black vomit; melena; temperature 37°C.; pulse 80; albumin ++. Apr. 27. Temperature 36.6°; pulse 66; albumin ++. Apr. 28. Temperature 37°; pulse 80; albumin ++. Recovery.

Apr. 26 (4th day). Blood taken for cultures. Dark-field examination of cultures 11 days after they were made failed to reveal any leptospira. Six guinea pigs inoculated with the 10 day culture material from this case also yielded negative results.

Case 14 (Severe; Recovered).—F. N., female, age 25 years; native of Morropon. Apr. 25, 1920, 11 a.m. Onset. Apr. 26. Temperature 38.5°C.; pulse 114; albumin trace; headache; backache; muscular pain; face flushed; conjunctivæ congested. Apr. 27. Temperature in morning 39.2°; pulse 106; albumin +. 4 p.m. Temperature 39.5°; pulse 120; severe pain and weakness; nausea, but no vomiting. At request of patient 20 cc. of anti-*icteroides* serum brought from The Rockefeller Institute were injected intravenously. 7 p.m. Temperature 38.9°; pulse 100; stronger; pains relieved. Apr. 28. Afternoon temperature 38.2°; pulse 100; albumin ++; nausea; bilious vomit. Apr. 29. Temperature 38.2°; pulse 90; no nausea; albumin ++. Apr. 30. Temperature 37.9°; pulse 86; mild pharyngitis. May 1. Temperature 36.8°; pulse 80; albumin +; recovering.
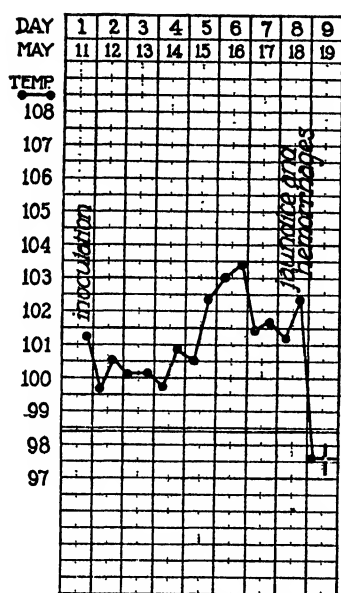
G.P. 39 (Strain 1 – Case 10).
Chart 1.
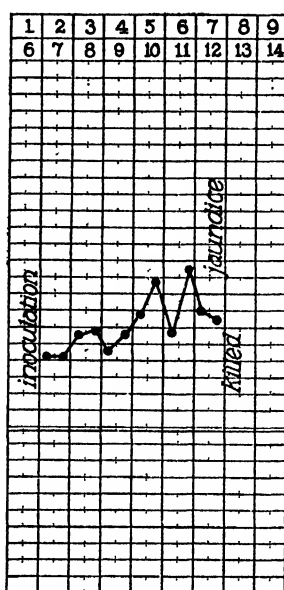
G.P. 26 (Strain 2 – Case 11).
Chart 2.
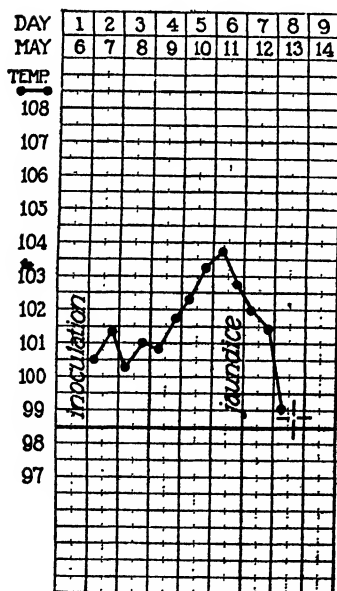
G.P. 2 (Strain 3 – Case 12).
Chart 3.
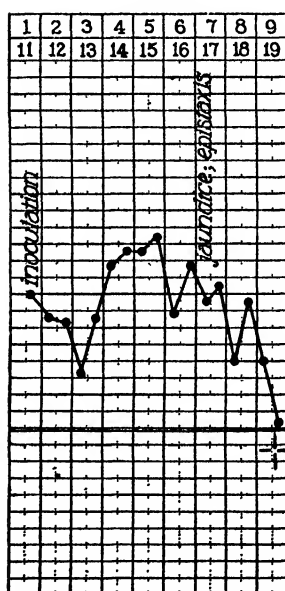
G.P. 45 (Strain 4 – Case 14).
Chart 4.

CHARTS 1 to 4. Temperature curves of guinea pigs inoculated with material from yellow fever cases in Peru.

Apr. 27 (48 hours after onset). Blood taken in the morning. The cultures were examined on May 8 when 11 days old, and living leptospiras were found in one tube, apparently dead ones in another.

May 6. Six guinea pigs (Nos. 7 to 12) were inoculated with the 9 day culture material. A typical infection was induced in three, while the other three remained apparently well. One of the latter showed hemorrhagic areas in the lungs when examined after 12 days. The other two had no macroscopic lesions, indicating that they were both completely refractory to this strain.

Transfer was made from one of the positive animals into three guinea pigs, all of which died with typical infections. Leptospiras were demonstrated in varying number in the blood as well as in the emulsions of liver and kidneys, and pure cultures were obtained from the blood of these animals. Chart 4 shows the temperature curve of one of the guinea pigs (No. 45) infected with Strain 4.

In Case 14, as in Cases 10, 11, and 12, positive transmission to guinea pigs was obtained by means of culture material. The initial cultures usually contained living leptospiras. In the blood and liver or kidneys of the infected animals the leptospiras were demonstrated, and a pure culture of *Leptospira icteroides* was recovered from the blood.

Identification of the Morropon Strains.

Upon our return to The Rockefeller Institute, we proceeded with the identification of the strains of leptospira isolated from yellow fever cases in Morropon along the lines previously followed. The Pfeiffer phenomenon was determined, as well as the effects of immune serums upon the organism *in vitro*.

Rich cultures of leptospira strains (Nos. 1 and 2) were employed in these experiments. The serums used were monovalent immune serums prepared in rabbits with Guayaquil Strain 1 of *Leptospira icteroides*. For purposes of control American Strain 2 of *Leptospira icterohæmorrhagiæ* was tested simultaneously. Moreover, a polyvalent immune serum prepared in a horse with several Guayaquil strains of *Leptospira icteroides* was also tested. The results obtained are given in Table I.

As Table I shows, the leptospira strains from Morropon gave positive Pfeiffer reactions with the immune serums prepared with the Guayaquil strains, but negative reactions with the anti-*icterohæmorrhagiæ* serum. Likewise, with respect to their behavior towards

these serums *in vitro* an indubitable specificity for the anti-*icteroides* serum is evident. The slight reaction with the anti-*icterohæmorrhagiæ* serum may be regarded as a group reaction among closely allied species. It is concluded, therefore, that the leptospiras isolated from Morropon and Guayaquil cases of yellow fever are of the same species.

TABLE I.

Identification of the Morropon Strains.

Mode of test.	Strain No.	Anti-*icteroides* serum (monovalent, rabbit).	Anti-*icteroides* serum (polyvalent, horse).	Anti-*icterohæmorrhagiæ* serum (monovalent, rabbit).	Controls without immune serum.
Pfeiffer (30 min.).	1	Complete disintegration (positive).	Complete disintegration (positive).	For the most part active (negative).	Very active (negative).
	2	Almost complete disintegration (positive).	Complete disintegration (positive).	For the most part active; few appear distorted (negative).	Very active (negative).
In vitro; allowed to stand for 18 hrs.	1	Complete agglutination; some motile leptospiras.	Complete agglutination and immobilization.	Slight agglutination and few immobilized.	Very active; no agglutination.
	2	Complete agglutination; some motile leptospiras.	Complete agglutination; few motile leptospiras.	Partial agglutination and immobilization.	Active; no agglutination.

DISCUSSION AND SUMMARY.

Fourteen typical cases of yellow fever were studied in northern Peru during an epidemic occurring in 1920, nine in Payta in March and April, and five in Morropon and Piura in April and May. The method of investigation was similar to that previously employed, but as the laboratory facilities were very meager certain changes were required. Although in Payta the work was handicapped by the lack of electric light, the scarcity of water and animal food, the unsuitability of the guinea pigs for inoculation, and the changes in culture media due to age, the results obtained under these adverse conditions were by no means negative. While in no instance was there a typical

infection produced in animals, either by direct inoculation of blood or with culture materials, yet certain guinea pigs in each series showed temporary febrile reactions or definite hemorrhagic lesions of the lungs indicative of a mild leptospira infection. Direct search for *Leptospira icteroides* in the blood of patients or in culture materials was not made because the dark-field microscope could not be used.

Subsequently, at Piura, the laboratory facilities were vastly improved, the use of the dark-field microscope was made possible by means of a storage battery, and a fresh stock of young healthy guinea pigs was received from New York, and fresh rabbit serum obtained in Piura. In the study of the materials obtained from five cases of yellow fever in Morropon all these added facilities were taken advantage of, with the result that the outcome was positive and convincing. Cultures from the five cases were examined after 11, 12, and 13 days, and in those from three cases living leptospiras were found.

By inoculation into suitable guinea pigs of culture material from these five cases, irrespective of whether or not leptospiras were detected under the dark-field microscope, a typical *Leptospira icteroides* infection was produced from four of the five cases. In one of these no leptospira had been detected in the culture tubes. Thus one case only yielded negative results, in that no leptospiras were found under the dark-field microscope and the animal inoculation was negative.

The leptospira was demonstrated in the blood or organ emulsions of the infected guinea pigs, and further transmission of each strain to other guinea pigs was obtained and pure cultures were secured.

A few points of practical significance appeared in the course of the present investigation. One is the importance of using fresh rabbit serum for culture media. Old rabbit serum, whether in pure form or incorporated with agar, etc., which had been kept for several months in a tropical climate, proved to be unsatisfactory for obtaining a growth of *Leptospira icteroides*. A second point of interest is the variation in susceptibility of guinea pigs to infection with *Leptospira icteroides*. In two of four series of positive animal inoculations with the Morropon culture materials only one-half of the guinea pigs inoculated with given materials developed typical symptoms. The other half either suffered from a transient mild infection, as evidenced by a few hemorrhagic foci in the lungs, or escaped infection altogether.

From these facts it is highly probable that the lung lesions and febrile reactions observed in certain guinea pigs inoculated with the Payta materials were due to a mild leptospira infection. In a comparative experiment the native guinea pigs procured in Payta were found to be more resistant to the leptospira infection than those recently brought from New York. In fact, only a small portion of the former succumbed to typical infection even when inoculated with a virulent strain of *Leptospira icteroides* obtained from the Morropon epidemic.

In conclusion it may be stated that of fourteen cases of yellow fever studied in Peru, a typical leptospira infection, together with the demonstration of the organism in experimentally infected guinea pigs, was obtained in four, while in the majority of instances indications of a mild, non-fatal leptospira infection were observed. In a few cases only were the results entirely negative.

The leptospira isolated from Morropon cases of yellow fever, which is morphologically and culturally identical with the Guayaquil and Merida strains of *Leptospira icteroides*, was also shown by immunity test to be indistinguishable from the Guayaquil organism.

IMMUNOLOGY OF THE PERUVIAN STRAINS OF LEPTOSPIRA ICTEROIDES.

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In the course of a study of the etiology of yellow fever in Peru,[1] an effort was made to solve several related problems of the disease. The first point taken up was the behavior of the serums of Peruvian convalescents in Pfeiffer's reaction towards strains of *Leptospira icteroides* isolated elsewhere; the second concerned the action, *in vitro* and *in vivo*, on the Peruvian strains of the leptospira, of a polyvalent immune serum prepared in horses with several strains of *Leptospira icteroides* of Guayaquil origin; the third related to the difference in the natural resistance to the leptospira infection of the native guinea pigs as compared with guinea pigs recently brought from New York; and the fourth and last point bore on the availability of old rabbit serum for cultivating *Leptospira icteroides*. The last two questions had arisen as a result of the rather unfavorable outcome of the work carried out in Payta, and, as will appear later, proved to be important.

Pfeiffer Reaction with Serum from Convalescents.

The method was similar to that previously employed. 1 cc. of each serum was mixed with 0.2 cc. of a rich 6 week *icteroides* culture of Guayaquil Strain 1 and the Merida strain. The guinea pigs used were those recently brought from New York. Observations were made at the end of 1 hour.[2]

Nine convalescents were accessible for this test. Of these, four came from the Payta (1920), four from the Piura (1919), and one from the Morropon (1920) epidemic. The serums were obtained in the five 1920 cases within a period ranging from 7 to 36 days from the time of onset of the fever and in the four 1919 cases at the end of 10 to 11 months. The results obtained are shown in Table I. The interpretation of the findings is evident. All the Peruvian cases, except two instances to be discussed below, gave a positive Pfeiffer reaction to two different strains of *Leptospira icteroides*, irrespective of whether the case occurred in Payta, Piura, or Morropon.

TABLE I.

Pfeiffer Reaction with Serum from Convalescents.

Serum No.	Locality of epidemic.	Length of time between onset of yellow fever and withdrawal of blood.	Strain of *Leptospira icteroides*.	Results.
		days		
1	Payta.	7	Guayaquil No. 1	Positive
2	"	7	" " 1	"
3	"	36	" " 1	"
4	"	17	" " 1	"
		mos.		
5	Piura.	11	Merida.	"
6	"	10	"	"
7	"	10	"	Doubtful.
8	"	10	"	Partial reaction.
		days		
9	Morropon.	21	"	Positive.
Control.			"	Negative.

The exceptions were the serums obtained from two persons who had had yellow fever 10 months previous to the time of testing, both giving a slight or partial reaction. It may be noted, however, that two other individuals who had had an attack 10 and 11 months previously reacted positively. The reason for a partial reaction in the first two cases probably relates to a gradual diminution of the antibodies responsible for the Pfeiffer reaction.

The foregoing observations are of value in establishing the identity of the organisms that were present in individuals suffering from yellow fever in Ecuador, Mexico, and Peru. The positive Pfeiffer

reaction with the serums of yellow fever convalescents in Payta also indicates that the guinea pigs of the Payta experiments which had shown suspicious febrile reactions and hemorrhagic lesions of the lungs and kidneys without developing a typical fatal infection, had actually suffered a mild infection with *Leptospira icteroides*. Finally, it may be added that the guinea pigs which were employed in the Pfeiffer tests and reacted positively showed no symptoms and survived, while the control animal (inoculated with the Merida strain) suffered from a typical but non-fatal infection.

Virulence.

The virulence of the Morropon strains was next tested on New York guinea pigs. The material used was an emulsion of kidney

TABLE II.

Determination of the Minimum Lethal Dose of Morropon Strains.

Amount of kidney emulsion in 1 cc.	Strain 1.		Strain 3.	
	Incubation period.	Result of inoculation.	Incubation period.	Result of inoculation.
cc.	*days*		*days*	
1	4	Died in 9 days.	No test made.	No test made.
0.1	4	" " 8 "	3	Died in 8 days.
0.01	3½ ·	" " 7 "	4	" " 9 "
0.001	5	Recovered.	3	" " 7 "
0.0001	6½	Died in 11 days.	5	" " 8 "
0.00001		No infection.	5	" " 10 "

The animals used for this experiment served at the same time as controls for the therapeutic experiments reported in Table III.

made in saline solution in a ratio of approximately 1 gm. to 10 cc. After the coarse tissue fragments had settled, the turbid supernatant portion was employed for inoculation. Ascending tenfold dilutions were prepared with 0.9 per cent saline solution, and 1 cc. was injected intraperitoneally into guinea pigs, one animal being used for each dilution. Table II gives the minimum lethal dose as determined for Morropon Strains 1 and 3. The approximate minimum lethal dose for Strain 1 lies between 0.0001 and 0.00001 cc., and that of Strain 3 beyond 0.00001 cc. The grade of virulence of the strains is, therefore, about the same as that of strains previously studied.

Therapeutic Experiments with Anti-icteroides Serum.

The reaction of the Peruvian strains towards the anti-*icteroides* immune serum prepared with the Guayaquil strains confirms the other evidence of the identity of the organisms. It has been shown in our first paper[1] that the leptospira from Peru gave a positive Pfeiffer reaction with this immune serum. In the present series of experiments the object was to determine, in guinea pigs given multiple minimum lethal doses of Morropon Strains 1 and 3, the prophylactic and curative values of a specific polyvalent anti-*icteroides* immune horse serum. The immune serum was administered in varying quantities and at different periods after inoculation, even after the appearance of symptoms, up to the time when the animals were fast approaching the terminal stage of the disease induced. The guinea pig kidney emulsions used for infecting the animals were injected intraperitoneally in uniform amounts of 0.2 cc., this dose representing at least 2,000 minimum lethal doses of Strain 1 and 20,000 minimum lethal doses of Strain 3. The immune serum was given intraperitoneally also in doses of 0.00001, 0.0001, 0.001, 0.01, and 0.1 cc., contained in saline solution to a total volume of 1 cc., and finally 1 cc. of undiluted serum. In order to obviate the factor introduced by an occasional unusually refractory animal, more than two of the recently brought guinea pigs were used in testing the effect of each dose of serum. Table III, which summarizes the results, brings out the following points. (*a*) The anti-*icteroides* serum is capable of checking the development of the infection, provided a sufficiently large quantity is given during the incubation period. The four deaths which took place among animals treated during this period occurred among those which did not receive serum until 72 hours after inoculation; *i.e.*, at the end of the incubation period. (*b*) A quantity of serum larger than 0.01 cc., given during the febrile stage (96 hours after inoculation) usually prevented the infection from reaching the icteric stage. This was true particularly with the animals infected with Strain 1, which had scarcely a tenth of the virulence of Strain 3. (*c*) The effect of the serum became uncertain when it was given during the icteric stage (5 to 6 days after inoculation). With 0.01 cc. no animal was saved, while 0.1 cc. saved three out of

four infected with Strain 3, but none with Strain 1. 1 cc. of undiluted serum saved one out of three with Strain 1 and three out of four with Strain 3. That the proportion of recoveries among the animals treated during the icteric stage does not correspond with the virulence of the strains can be explained only on the basis of the natural resistance of individual guinea pigs; at all events, death occurred in both groups, even when 1 cc. of serum was given.

TABLE III.

Protective and Curative Effect of Anti-icteroides Serum in Guinea Pigs Experimentally Infected with Leptospira icteroides.

Strain.	Amount of serum.	Animals treated before beginning of fever.			Animals treated during febrile stage.			Animals treated after jaundice appeared.		
		Total.	Remained well.	Died.	Total.	Recovered without jaundice.	Died.	Total.	Recovered.	Died.
	cc.									
0.2 cc. of Strain 1 culture, representing 2,000 minimum lethal doses.	0.0001	2	1	1						
	0.001	5	5	0	3	0	3			
	0.01	4	4	0	5	5	0	2	0	2
	0.1	4	4	0	3	3	0	2	0	2
	1				4	4	0	3	1	2
0.2 cc. of Strain 3 culture, representing 20,000 minimum lethal doses.	0.0001	2	2	0						
	0.001	6	5	1	2	0	2			
	0.01	6	4	2	5	3	2	2	0	2
	0.1	3	3	0	3	3	0	4	3	1
	1				2	2	0	4	3	1

The controls without serum treatment are represented by the animals of the experiment recorded in Table II.

Resistance of Native and Imported Guinea Pigs to Leptospira icteroides.

Certain tests made with guinea pigs obtained in Payta suggested that the native Peruvian guinea pigs are more refractory to the leptospira than guinea pigs brought from New York. To determine this point four groups of guinea pigs were inoculated, on May 16, 1920, with a uniform amount of kidney emulsion from a guinea pig which had shown a typical infection with Morropon Strain 4. The

animals inoculated comprised: (1) fifteen native guinea pigs which had once been inoculated with culture materials in Payta, without any definite infection having been induced; (2) six native guinea pigs which had not been used; (3) eight American guinea pigs which had formerly been inoculated with culture materials in Payta, without infection having been induced; and (4) four newly imported American guinea pigs. Table IV summarizes the result. It will be noted that the virulence and dose of the strain used were such that even the young American guinea pigs, used as a standard of susceptibility, did not all develop a fatal infection. This effect was rather an advantage, since, had multiple fatal doses been given, the difference in susceptibility might not have come out so clearly.

TABLE IV.

Relative Resistance of Native and American Guinea Pigs to Leptospira icteroides.

Guinea pigs.	Total No. injected.	Escaped infection (no jaundice).	Animals showing definite infection.*	
			Recovered.	Died.
Native (once used) ?.....................	15	9	1	5
. " (normal)........................	6	6		
American (old lot; once used).............	8	3	3	2
" (new lot; normal)..............	4	0	2	2

* This includes guinea pigs which showed typical lesions when killed.

The striking feature of the foregoing experiment is the complete resistance of all six normal native guinea pigs in contrast to the four normal American animals, of which two, at least, died of a typical infection. Among the animals inoculated on a former occasion, both the native and the American groups contained susceptible as well as refractory ones, the difference being that a larger proportion of the native (60 per cent) than of the American (37.5 per cent) proved resistant.

The relatively large proportion of resistant animals among the native guinea pigs, as well as among the American guinea pigs which had withstood the unfavorable climate and other hardships, is a point to be taken into account in future work.

Deterioration of Rabbit Serum through Age.

That an alteration takes place in rabbit serum when it is subjected to long transportation in a hot climate is evident from the fact that no growth of *Leptospira icteroides* could be obtained with such serum. On the other hand, old rabbit serum which has been kept in the refrigerator up to 6 weeks has been found still suitable for making subcultures of *icteroides*. Whether the same would be true after 2 or 3 months standing at 4°C. has not, however, been determined.

The rabbit serum which was used in the cultivation experiments in Payta[1] was 3 months old and had been exposed to the ordinary tropical temperature of the region, which is sometimes 40°C. during the day, and it contained considerable precipitate. It was thought probable that the deterioration of the serum through age and climatic conditions might have been a factor in the unsuccessful outcome of the Payta experiments. Moreover, another lot of serum, which had been collected in New York in April and hermetically sealed, was brought down to Piura under ordinary temperature conditions. Some tubes contained precipitate, while others had remained clear. It was used when 50 days old for making subcultures of four Morropon strains of the leptospira, but when the culture tubes were examined 10 days later no growth could be found. Fearing a possible loss of all strains, we made two more sets of subcultures of the same strains, one set with the old and another with fresh rabbit serum. With the media containing the fresh rabbit serum a rich growth took place with three of the four strains, but there was no growth in the tubes prepared with the old serum.

Other factors, however, made it difficult to determine how important it is in general to employ fresh serum in culture work with *icteroides*. The Peruvian strains were unusual in that they early degenerated in culture. The initial cultures from human cases never became very rich, and they disappeared within about 3 weeks. The first generation cultures from the infected guinea pigs grew fairly well within a week, but at the end of 2 weeks examination showed only a few active organisms and many degenerating. Of the cultures of Strain 3, none was alive, and injection into guinea pigs failed to recover it. The remaining strains (Nos. 1, 2, and 4) were successfully

subcultured by using fresh rabbit serum, as noted above. It is certain that all the strains would have been lost if we had relied entirely on the old serum. The present study does not indicate whether the importance of using fresh serum applies only to the primary human and recently isolated generations or even to remote subcultures.

SUMMARY.

Serum from yellow fever convalescents from Payta, Piura, and Morropon gave a positive Pfeiffer reaction with the strains of *Leptospira icteroides* isolated in Guayaquil and Merida. The serum also protected the guinea pigs from these strains in the majority of instances. The Pfeiffer reaction was complete with all recent convalescents (7 to 36 days) but slight or partial in some instances with serum derived from individuals who had had the attack of yellow fever 10 months previously.

The virulence of the Morropon strains was found to be approximately the same as that of the Guayaquil or Merida strains. With one strain the minimum lethal dose for the guinea pig was less than 0.00001 cc. of a kidney emulsion from an infected guinea pig.

. Suitable quantities of the anti-*icteroides* serum administered to guinea pigs inoculated with 2,000 to 20,000 minimum lethal doses of infective material prevented the development of the infection, or a fatal outcome, according as the serum was given during the incubation period or after fever had appeared. The earlier the administration of the serum the smaller was the quantity needed; during the incubation period 0.0001 to 0.001 cc. was sufficient, during the febrile period 0.01 to 0.1 cc. was required to check the progress of the disease, and even at the time when jaundice had already appeared, the injection of 0.1 to 1 cc. saved three out of four animals inoculated with Strain 3 and one out of three inoculated with Strain 1.

The native guinea pigs secured in Payta proved to be unusually refractory to infection with *Leptospira icteroides* as compared with normal guinea pigs recently imported from New York.

Fresh rabbit serum is recommended for culture work with *Leptospira icteroides*.

OBSERVATIONS ON THE DISTRIBUTION AND HABITS OF THE BLIND TEXAN CAVE SALAMANDER, TYPHLOMOLGE RATHBUNI.*

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When in 1895 the artesian well was drilled at the U. S. Fish Hatchery in San Marcos, Texas, the first specimens known to biologists of the blind cave salamander, *Typhlomolge rathbuni,* were brought up with the waters from the depths of the ground. The animals were described by Prof. L. Stejneger. For several years after this a relatively large number of the blind salamanders, about 100 a year, were found in the basin of the well, but gradually the number decreased and lately has been reduced to a few specimens a year.

When the question arose of subjecting this animal to certain experiments on metamorphosis, it became evident that a number of specimens sufficiently large for this purpose could be obtained only through an extensive search in the actual habitat of the *Typhlomolge.* With the aid of a special grant from The Rockefeller Institute for Medical Research an extensive study of the caves of San Marcos and environment was made by the writer and Mr. C. A. Campbell, at that time instructor in biology at Coronal Institute in San Marcos, during the months of August and September, 1916. So far as the number of animals obtained is concerned, the result was disappointing. But, on the other hand, several observations were made which seem to be of interest as regards the distribution and habits of the *Typhlomolge* and which furnish valuable suggestions as to the methods which must be employed in order to procure a large number of animals. The writer hopes to stimulate a search for these salamanders on a large scale, in order to make this interesting form accessible to the experimental biologist who is in need of just such an animal as *Typhlomolge rathbuni* for attacking many important problems.

GENERAL CHARACTERS OF THE REGION.

As pointed out, the specimens described first by Stejneger were found in the basin of the Artesian Well in the Fish Hatchery in San Marcos, and were carried up into this basin by the flowing water of the Artesian Well. During a two months' stay in San Marcos, we secured only two specimens from this basin, but five other specimens were found in three other localities, i.e., in Frank Johnson's Well, in Ezell's Cave and in Beaver Cave.

In order to understand the conditions which might have led to the present distribution of *Typhlomolge* and because these conditions in the future may be an important guide in tracing the subterranean channels which the animals inhabit, a careful study was undertaken. It was found that the conditions in the three places where we found *Typhlomolge* are essentially similar to those existing in the locality from which the water of the San Marcos Artesian Well is derived.

Before describing the well and the caves in which we found *Typhlomolge* it is necessary to point out the geologic peculiarities of this area of Texas, since these conditions not only led to the formation of the caves but also to the present distribution of the *Typhlomolge*. Whether or not they are also responsible for the peculiar characteristics of the animal as Eigenmann and Stejneger assume, is an important question, the answer to which, however, cannot be given before extensive experiments on this species have been carried out.

San Marcos is located on the so-called Balcones scarp line. This line runs from Austin to Del Rio in a south-westerly direction and separates in a most distinct way the Edwards Plateau (north of the line) from the Rio Grande Plain (south of the line). It forms the escarpments of the plateau towards the plains. Along this line a faulting has taken place in Eocene time (Hill and Vaughan, p. 260), during which the part that now forms the plain was thrown down and the northern part which now constitutes the plateau was left behind. In consequence of this faulting, any particular geologic stratum now lies deeper on the side thrown down than on the plateau.

It was apparently this faulting which has led to the formation of many cracks in the rock layers. The caves near the escarpments of the Edwards Plateau represent gigantic cracks. Besides this

factor there is still another cause leading to the formation of caves in this region. The entire area of the Edwards Plateau constitutes a huge outcrop of the Cretaceous. In the soft strata of the various cretaceous formations of the plateau, numerous caves have been formed by the mechanical force of the water combined with its dissolving action. By this process most of the rivers of the Edwards Plateau have disappeared almost entirely from the surface, and their former beds are dry. These rivers have sunken beneath the surface where they flow in subterranean channels.

FIG. 1. Basin of the Artesian Well of the U. S. Fish Hatchery in San Marcos.

THE SAN MARCOS ARTESIAN WELL.

When the artesian well of the U. S. Fish Hatchery in San Marcos (Fig. 1) was drilled in 1895, a number of water reservoirs were opened up by the drill. At present only the water is used which rises from a depth of approximately 190 feet. Here a cave filled with water was opened up; in it the *Typhlomolge* lived. The water in this cave must have been under a pressure sufficiently high to carry it up 190 feet.

The *Typhlomolge*, thus, lived most abundantly in water under high pressure and without any access to air except that present in the water. The water of this cave belongs to the so-called "sweet water" horizon of the Edwards limestone in which formation the cave is located.

We measured the temperature of the water as it comes out of the tube of the well as approximately 21.5° C. Among the fauna of the cave from which the water of the San Marcos Well rises, are particularly conspicuous two crustaceans, both unpigmented and eyeless, an isopod, *Cirolanides texensis* and decapod, *Palæmonetes antrorum*. The latter species is of particular importance, since so far it has been found to occur in all localities which are inhabited by the, blind salamanders.

The cave of the San Marcos Well, thus, is characterized in the following manner: (1) It is situated in the Edwards limestone. (2) It contains water dirived from the "sweet water" horizon. (3) The temperature of the water is approximately 21.5° C. (4) The water is inhabited by the decapod, *Palæmonetes antrorum*.

FRANK JOHNSON'S WELL.

Approximately two miles southwest of the San Marcos court house (see map, Fig. 2), the flat valley of the dry Purgatory Creek crosses the Balcones scarp line opening here into the flat valley of the San Marcos River. Its northern slopes are formed here by the San Marcos Hill. Purgatory Creek originates near the Devil's Backbone, the divide between the Guadalupe and Blanco Rivers, at Boyett's Farm, about 14 miles northwest of San Marcos. It is dry at present, but several of the older inhabitants claim that this creek had running water in it until about 50 years ago. At present only a few water holes are left in the upper course of the valley and several sink holes have formed in its lower course. These are filled temporarily with rain water. In time of severe cloud bursts the water in the creek becomes a torrent rising to a height of 8 feet, but it disappears completely within several hours.[1] Purgatory Creek has now become a

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[1] The general character of a creek like this may be found described in Hill and Vaughan, page 207.

subterranean creek.   Mr. Frank Johnson's farm is located near where
the creek crosses the fault line.

Mr. Johnson informed me soon after my arrival in San Marcos that
the blind white salamander has been seen in his well, and in fact this
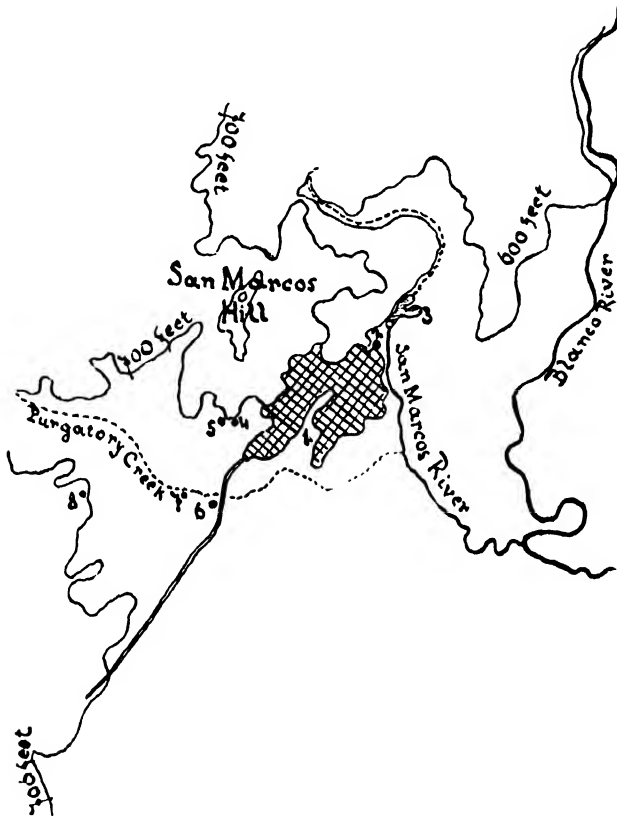
FIG. 2. Map of San Marcos Area.

1. San Marcos.
2. Artesian Well of U. S. Fish
   Hatchery.
3. Head of San Marcos River.
4. Beaver Cave.

5. Ezell's Cave.
6. Frank Johnson's House.
7. Frank Johnson's Well.
8. Swift's Cave.

well has yielded us more salamanders than any other place.   It is
shown in Fig. 3.

The well is located in the valley of Purgatory Creek, a short distance
above where the creek enters the plain.   Part of the flat valley is

visible in the figure. Near the well is a sink hole (Driskel's Water Hole, see diagram, Fig. 4). The well was dug from a level of 613 feet[2] above sea (San Marcos Court House 620 feet) to a depth of $31\frac{2}{3}$ feet. There a cave was struck which now communicates with the well through a slit in the well bottom, as indicated in the diagram (Fig. 4). From this slit the water rose to from 3 to 5 feet in the well. This makes the surface altitude of the water about 584 feet. Mr.
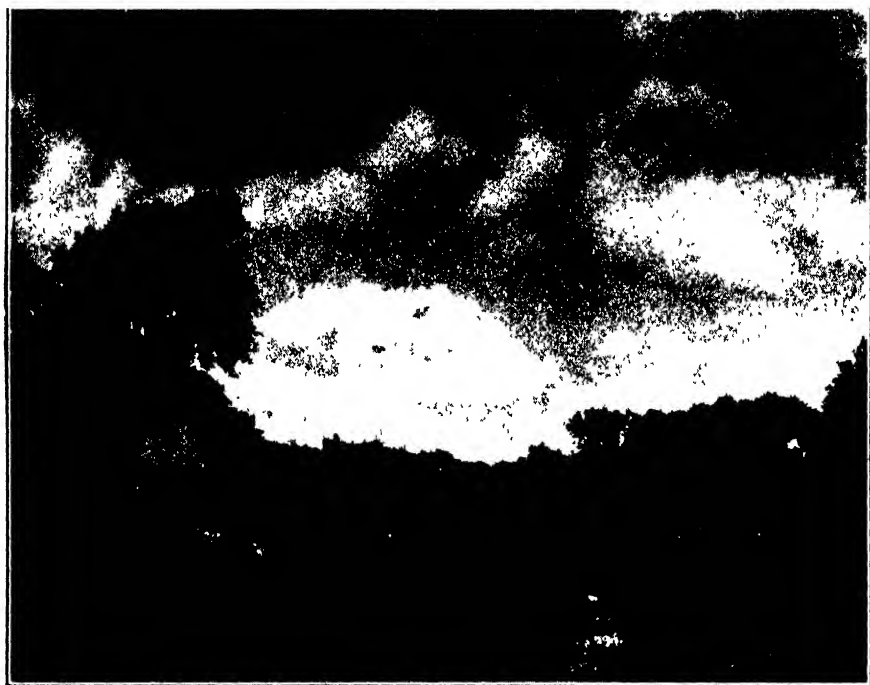
FIG. 3. Frank Johnson's Well. Showing the well house and at the right of the well house the dry and flat valley of the Purgatory Creek.

Johnson claims that the water is flowing. There is no doubt that Johnson's Well communicates with the subterranean Purgatory Creek. As in the case of the San Marcos Artesian Well, the water in this completely water filled cave must have been under a pressure

[2] Altitudes above sea level were measured by means of an anaeroid barometer and therefore are only approximately correct (within several feet). Dimensions other than altitudes were measured directly, except when otherwise stated.

sufficiently high to lift it to 3 feet in the well.   It again is evident that the *Typhlomolge* prefer to live in water under high pressure and in caves which are filled entirely with water.   The water of Johnson's Well has the same temperature as that of the San Marcos Artesian Well and also has the same taste.   Besides the *Typhlomolge*, Frank Johnson's Well contains also the *Palæmonetes antrorum* and the *Cirolanides texensis*.

Thus, though the water of the Frank Johnson Well represents the subterranean Purgatory Creek, it shows great similarity to the water of the San Marcos Artesian Well.   Particularly the presence in Purgatory Creek of 3 species typical of the artesian well would suggest
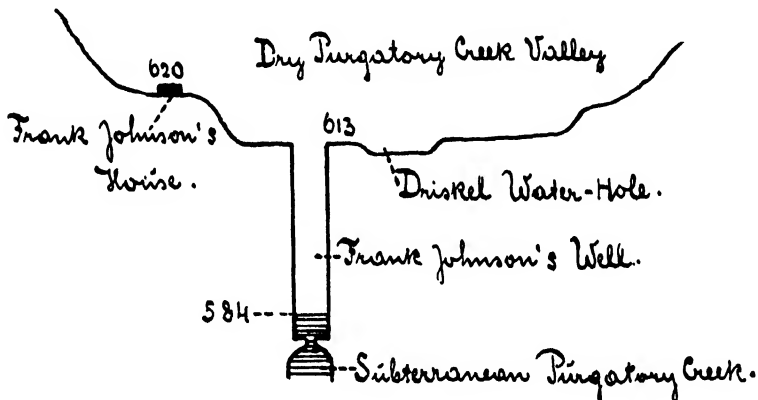
FIG. 4. Purgatory Creek Valley and Frank Johnson's Well.   Diagrammatic section reconstructed from several cross sections.   The figures indicate altitude above sea level in feet.

that in some way the Purgatory Creek water is in communication with the so-called sweet water horizon near the San Marcos Artesian Well.

In Frank Johnson's Well the *Typhlomolge* were seen to pass through the slit from the cave into the well.   During my stay in San Marcos, the water in the well was too high to catch the salamanders directly and for this reason traps were submerged in the well.   These were ordinary minnow traps.   In the beginning they were supplied with various kinds of bait, but in this way only the crustaceans mentioned above were caught.   The *Typhlomolge* did not seem to react to the bait, and later on when I observed the animals in the laboratory, it

became evident that the instinct of hunger is not sufficiently strong in the *Typhlomolge* to make them go into traps; it is in fact very difficult to make these animals eat. Later on the traps were placed with one opening directly in the slit; animals passing out from the slit had to go directly into the trap. In this way 2 *Typlohmolge* were caught in Johnson's Well, one in August, 1916, and another in September, 1916. After I had left, 11 more *Typhlomolge* were found by Mr. C. A. Campbell and Mr. Rufus Smith who from time to time looked after my traps. Thus, Frank Johnson's Well yielded us 13 specimens of *Typhlomolge*. They were caught as shown in the following table. The number is, however, too small to warrant any conclusions as to a possible influence of the season upon the frequency of the occurrence of *Typhlomolge*.

August,   1916.... 1
September, 1916. 1
November, 1916....... 2
December, 1916............. 1
January,   1917......... 2
April,   1917...... 1
Summer   1917..2
November, 1917.......................................3

One of the greatest difficulties encountered was to find a method of shipping the animals from San Marcos to New York; most of them did not survive the trip. In fact, only two ever reached the laboratory alive. The first seven specimens caught were taken on the train in a bucket filled with water. The jarring killed six. Among the eleven caught later on, only one survived the trip. Its safe transfer was accomplished by a fortunate incident. The animal was packed in a fruit preserving jar filled entirely with water and shipped in the winter. On arrival it was frozen tightly in a block of ice. This animal survived for one year in the laboratory. The only thing it could be made to eat were newly hatched larvæ of *Ambystoma macu-latum*. Though kept for most of the time in a dark room, the skin which in the beginning was white with a bluish, mother-of-pearl gleam, had darkened somewhat.

It should be pointed out here that slow reaction to food as exhibited by the *Typhlomolge*[3] is noteworthy in regard to certain findings of Miss E. T. Emmerson, who claims, upon anatomical reasons, a close relationship between *Typhomolge* and the larvæ of *Eurycea rubra*. We are keeping a large number of such larvæ in the laboratory and contrary to my experience with the larvæ of *Ambystoma* and other salamander larvæ, these larvæ react very slowly to food. In fact, it is impossible to make them eat every day aside from the fact that most of the individuals of this species will eat only at night.

EZELL'S CAVE.

Ezell's Cave was opened up several years before the San Marcos Well was drilled. The entrance to the cave is located on the southwest slope of the San Marcos Hill (see map, Fig. 2), where it slopes down to the valley of Purgatory Creek about 2 miles W.S.W. of the San Marcos Court House, and not far from a little ravine, the bed of the dry City Boundary Creek, a tributary to Purgatory Creek. This location of Ezell's Cave indicates that it belongs to the Purgatory Creek System, the river found in it probably being the subterranean course of the City Boundary Creek.

Ezell's Cave distinctly exhibits the aspect of a large crack in the strata of the hill, brought about by dislocation of the strata towards the Purgatory Creek Valley. The entrance to the cave (approximately 670 feet above sea level) is part of a 62 ft. slit in the surface (Fig. 5), which for the most part is closed up by large rocks and runs from N.N.W. to S.S.E., that being the direction of the long axis of all the various parts of the cave. As the diagrammatic cross and longitudinal sections (Figs. 6 and 7) indicate, the entire slit so far as accessible is divided into two compartments by means of the rock masses which were thrown down during the process of dislocation and following corrosion. These masses of debris form the bottom of the first story and in the N.N.W. corner leave open a small hole ("entrance hole"), $2\frac{1}{4}$ feet wide through which a narrow canal ("tube") may be reached which after running along the main axis of the slit for a short

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[3] Normann, who kept a specimen of *Typhlomolge* in captivity, also reports great difficulty in making the animal eat.

distance leads down into the second story or water room. This compartment of the cave contains a large body of water (Fig. 8).

This pond is not formed by water which drains through the strata forming the roof of the cave nor by water flowing directly into the
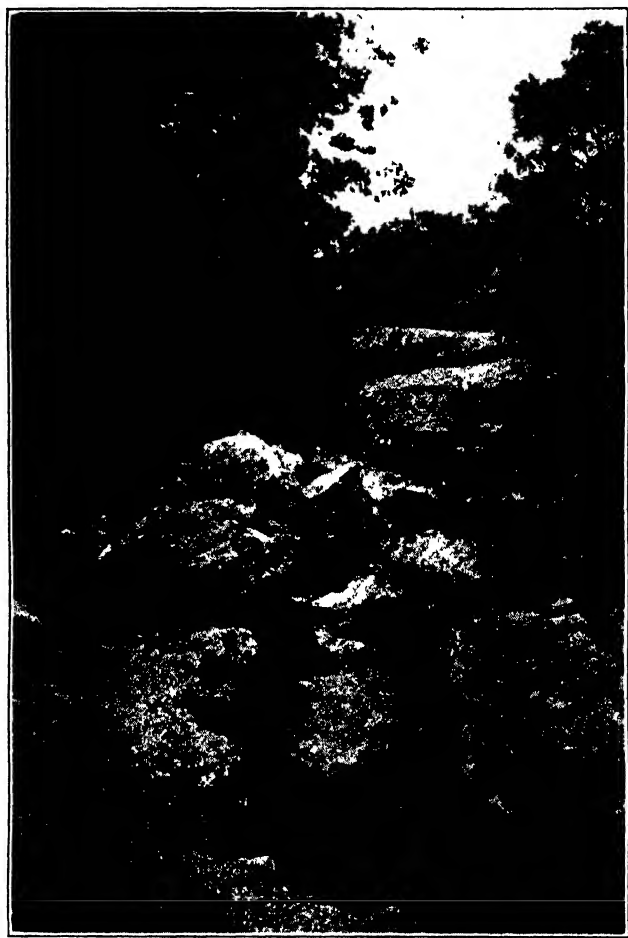
FIG. 5. Entrance to Ezell's Cave.

entrance of the cave, as the slope of the hill is drained in the course of rain. The pond is formed by a subterranean river, which is evident from the fact that the water is flowing, though hardly in a perceptible manner. The flow can be observed from the dislocation of bodies dropped into the water at the N.N.W. end of the pond. If the water

is not disturbed such bodies will arrive, in the course of an hour or so, at the S.S.E. end, thus indicating the direction of the flow. By means of a collapsible boat which was brought down into the water it is possible to follow the course of the subterranean creek towards N.N.W., (Fig. 9) for a distance of about $91\frac{1}{3}$ feet. The crack extends, however, beyond this point and by climbing over a number of rocks
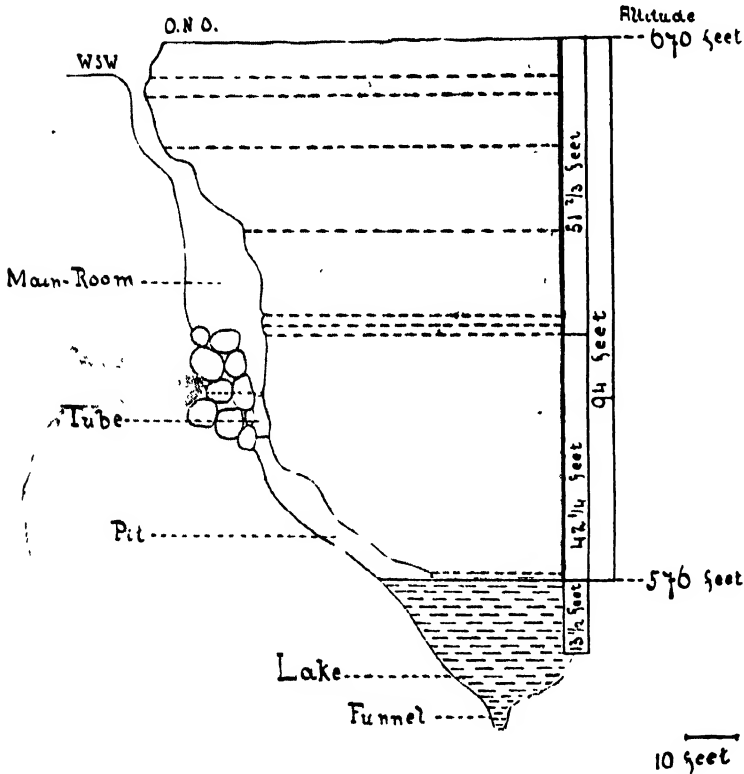
FIG. 6. Ezell's Cave. Diagrammatic section reconstructed from several cross-sections.

the creek can be seen to continue in this crack. But we had no opportunity so far to explore this part of the cave.

The greatest depth of the water is $13\frac{1}{2}$ feet, as far as it can be measured. It is, however, not possible to ascertain exactly the depth of the water and of the crack, since the water is covered in part by the overlapping wall of the crack forming a ledge over the water

(diagram Fig. 6 and photograph Fig. 9).   Underneath this ledge the ground can be seen to slope down very deeply; it is possible by means of a strong light to see a funnel shaped crater opening at the deepest part of the lake in which no bottom can be seen.

The entire crack, with the water which it contains, is located in the Edwards limestone; but as pointed out above, the structure of the cave would indicate that this crack may extend into the deeper lying strata.
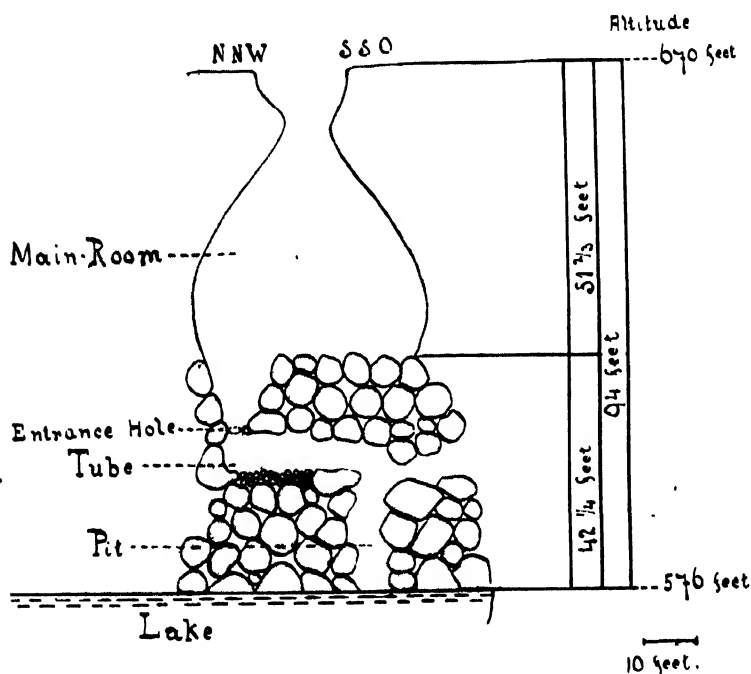
FIG. 7. Ezell's Cave.   Diagrammatic section reconstructed from several longitudinal sections.

The distance from the entrance down to the water surface is 94 feet, which makes the level of the water about 577 feet.   The altitude above sea level of the entrance of the cave was measured merely by means of a barometer, but the figure approaches the altitude of the water surface in Frank Johnson's Well near enough; the water levels in Frank Johnson's Well and in Ezell's Cave are approximately equally high.

The water is of an extreme clearness and of bluish color, typical also of the water of the sweet water system.   It also tastes like this water and has the same temperature (21.5°).   Using a sufficiently strong light one discovers immediately a great number of *Palæmonetes antrorum* swimming near the surface of the water, which thus contains also the same species of animals as were found in the water of the San Marcos Artesian Well and in Frank Johnson's Well.
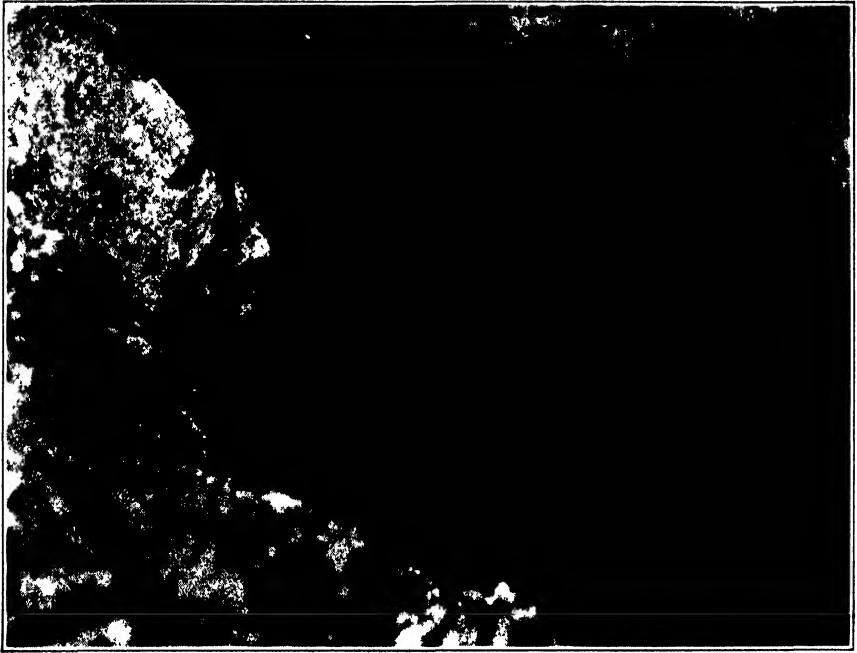
FIG. 8. Water-room in Ezell's Cave.

Hence the water in Frank Johnson's Well and that in Ezell's Cave have a number of characteristics in common.   They have the same taste, are of the same temperature, and their levels are equally high. They harbor the same species of animals.   From their characteristics and from their location it seems that they are parts of the subterranean Purgatory Creek System.

Furthermore, both of these water bodies have certain most conspicuous characteristics in common with the water of the San Marcos Artesian Well.   They are of the same temperature and contain the

same fauna.   One naturally would think of a direct communication between the Purgatory Creek System and the caves which supply the San Marcos Artesian Well.
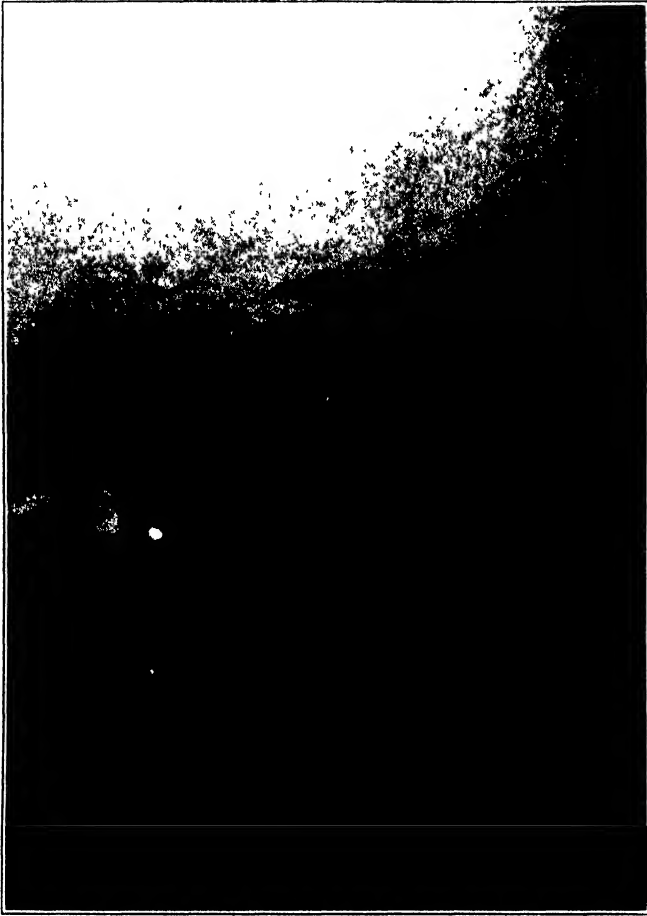
FIG. 9. Ezell's Cave Lake.   Showing the overlapping ledge.

We caught only one animal (78.5 mm.) in Ezell's Cave.   It was sitting quietly near the bank of the river where the water is shallow and did not seem to mind pebbles dropped down into the water near it, nor the glare of the light from two Columbia dry cells.   We spent 12 days in the cave under the most varied conditions, and conducted a most extensive search for *Typhlomolge*.   Hence the scarcity of this

species is somewhat perplexing.   It is possible that the animals prefer
to stay further down in the passages and cracks filled completely
with water under high pressure, an assumption which is supported
by the circumstances under which the animals were found in both the
artesian well and Frank Johnson's Well.   It may be that they rarely
and only by some incidental circumstances are induced to come to the
more open bodies of water.

So far as is known to the writer, the specimen of *Typhlomolge*
caught in Ezell's Cave in 1916 is the first and only one positively
known as having come from this locality.   But it is claimed by people
in San Marcos, as Mr. S. N. Stanfield, teacher of biology in the Texas
Normal School in San Marcos, informed me, that the first two *Ty-
phlomolge* ever seen were found in Ezell's Cave, $1\frac{1}{2}$ years before the
well was drilled, in a small boat which had sunk in Ezell's Cave Lake.

BEAVER CAVE.

Not far from the entrance of Ezell's Cave on the southwest slope
of San Marcos Hill and at an altitude of 652 feet above sea level,
near the dry bed of the City Boundary Creek is situated the entrance
to Beaver or Wonder Cave.   The location of the cave would indicate
that it belongs, like Ezell's Cave, to the Purgatory Creek System.

Beaver Cave represents the aspect of a straight running crack in
the strata of the Edwards limestone, the same as Ezell's Cave; this
crack, in part, has been widened out and its walls have been smoothed
down by the action of the water (Fig. 10).   Its bottom is made up of
huge masses of broken-down rocks which form, at some places, high
cliffs and rock masses, dividing the entire cave horizontally in a
number of rooms connected by narrower tubes with one another,
and vertically into several compartments.   Fig. 11 represents a
diagrammatic longitudinal section through the cave, which gives an
idea of the construction of this cave.   In Fig. 10, which was taken
parallel to the longitudinal axis, the slitlike shape of the cave is shown;
it can also be seen how smooth the walls have been washed by the
water entering easily through the thin roof of the cave.

The longitudinal axis of Beaver Cave runs from N.N.E. to S.S.W.,
forming an angle of approximately 25° with the longitudinal axis of

Ezell's Cave; the length of the entire slit is nearly 500 feet. It is claimed that there is a direct connection between Beaver Cave and Ezell's Cave.   We could not verify this statement, and it seems certain no one has actually found a connection. We found that at x in

FIG. 10. Interior of Beaver Cave. Photograph taken from rock 34 towards board rock. In back of the right hand side wall at its lower end, the opening of the "tube" is visible.

room VI. (see Fig. 11) a number of tightly packed rocks and masses of gravel make further penetration impossible at present and that the location of both caves and the direction of their main axes are not in favor of the statement mentioned above.

The deepest depression in the bottom of Beaver Cave is found in the room indicated in the diagram Fig. 11 as "Well-Room." The bottom of this floor is 62 feet below the surface and therefore at a level of 590 feet above sea level. As seen from the height of the water level in Johnson's Well and Ezell's Cave, no water of the Purgatory Creek System should be present in Beaver Cave. And in fact when the cave was discovered there was no water found. But a well drilling made at that time from the surface above the Well Room had indicated the presence of water only a few feet beneath the bottom of the Well Room. Therefore, a hole was dug in the bottom of the Well Room which led to water at a depth of about 3 feet or at the same level as the surface of the water in Ezell's Cave and Frank Johnson's Well (see Fig. 13).

At present one finds in the Well Room of Beaver Cave a rectangular basin approximately 6 feet in length, 3 feet in width and 6 feet in depth, the bottom of which is covered with mud and rocks, and the walls of which are lined with logs. This basin is filled with water half of its depth. Hence the surface of the water stands at the same level with the surface of the water in Frank Johnson's Well, and the suggestion seems justified that in this basin again part of the Purgatory Creek System was opened up. The water has the same taste as the water of the other localities mentioned and also has the same temperature (21.5° C.). In which way, however, this basin in Beaver Cave could be connected with the other localities cannot be stated
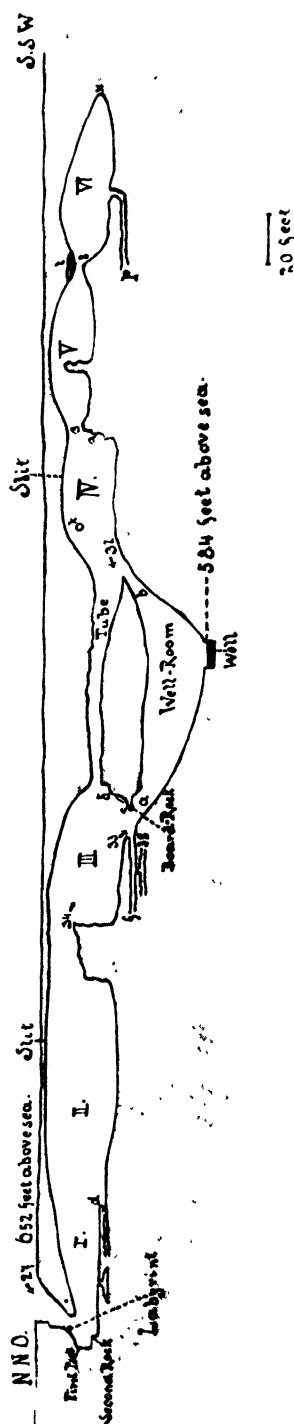
Fig. 11. Beaver Cave. Diagrammatic section reconstructed from several longitudinal sections.

with certainty at present, since the log lining of the wall made it impossible to search more closely whether or not the rocks of the wall contain any larger cracks or crevices. It also was not determined whether the water is flowing. But its clearness and the fact that the mud when stirred up disappears in a relatively short time would speak in favor of a slight current in the water. There is, however, one fact which hardly could be explained in any other way than that the water in the basin must be in connection at least at certain times with some larger bodies of water. The well in Beaver Cave contains both the *Palæmonetes antrorum* and the *Cirolanides texensis*, animals the transmission of which to the basin since it was constructed must have taken place by means of water currents which drive water from certain water bodies (harboring these animals) through the well.

Hence it is most probable that the water of Frank Johnson's Well, of Ezell's Cave and of Beaver Cave is the water of the subterranean Purgatory Creek System.

In the well of Beaver Cave two *Typhlomolge* were caught, one by means of a dip-net, the other in a trap which was laid with its opening just in front of a hole into which the animal had been seen to pass. One specimen was 82 mm. in length, the other one the largest caught measured 120 mm. Both these animals were observed for some time before they were actually caught; they proceeded to move in characteristic fashion—as described very accurately by Normann—by intermittent walking and resting in the presence of light. Even when the rays fell directly upon them, they did not seem to be disturbed. In this respect our observations made in the animal's natural habitats, agree very well with the observations made by Normann in the laboratory. Pebbles and a pocket-knife dropped into the water near the animal did not change its behavior; we have not found that the Typhlomolge as Normann claims possesses a specially high sensitivity towards disturbances of the water. Once stirred up the animals immediately swim towards the walls, and if they cannot find cover immediately, they swim along the wall toward the surface pushing out their snouts above the surface.

Before I was acquainted well enough with the general situation in the localities in question and before I had other facts indicating a possible connection between Beaver Cave and the Purgatory Creek, the occur-

rence of the *Typhlomolge* in the Beaver Cave well was puzzling, since it seemed to be difficult to explain how they could have been transferred to the well. In an anatomical study performed on *Typhlomolge rathbuni*, E. T. Emmerson points out the close relationship existing between *Typhlomolge* and *Eurycea* (*Spelerpes*), in particular *Eurycea rubra* and suggests that *Typhlomolge* may be the larva of an unknown species of the genus *Eurycea*. The writer of this article has a large number of larvæ of *Eurycea rubra* under observation and finds that in certain habits (feeding and especially the pushing out of the snout above the water when aroused) a remarkable resemblance exists between *Typhlomolge* and *Eurycea rubra*, a resemblance which was not observed by the writer in larvæ of the many other species of salamander closely watched in the laboratory. Concerning, however, the assumption that *Typhlomolge* is the larva of some species of *Eurycea*, this meets with one difficulty if it should mean that this species is still in existence. Ezell's Cave and especially Beaver Cave were closely searched for the presence of other salamanders. None were found in Ezell's Cave. In Beaver Cave, however, Mr. Campbell found about 20 specimens all belonging to the species *Plethodon glutinosus*; this is the only salamander which we could detect in these and other caves of the area around San Marcos. In view of this fact it appears that the suggestion as to whether or not *Typhlomolge* is the larva of a species represented at the present time also by metamorphosed specimens would be hardly more than speculation. It is, however, certain that it would be of the greatest value to raise the *Typhlomolge*, in order to study closely their mode of propagation, development and to subject these animals to certain experiments indicated by our present technic in the study of the metamorphosis of other salamanders.

In connection with the metamorphosis of *Typhlomolge* it may be pointed out that Miss Emmerson has made a statement which is so important that it arouses curiosity as to why it has attracted so little attention. Miss Emmerson searching for the organs of internal secretion of *Typhlomolge* found that the animal possesses a thymus gland, but she could not find a thyroid gland. If the lack of a thyroid gland could be confirmed—and we are preparing some of our specimens for examination with that end in view—Miss Emmerson's dis-

covery will explain why the *Typhlomolge* cannot metamorphose at present, since Allen has demonstrated that larvæ of frogs and toads whose thyroids were extirpated did not metamorphose, though the controls with intact thyroids all metamorphosed. Do the Proteidæ (*Proteus*) possess thyroids, is the lack of the gland common to all of
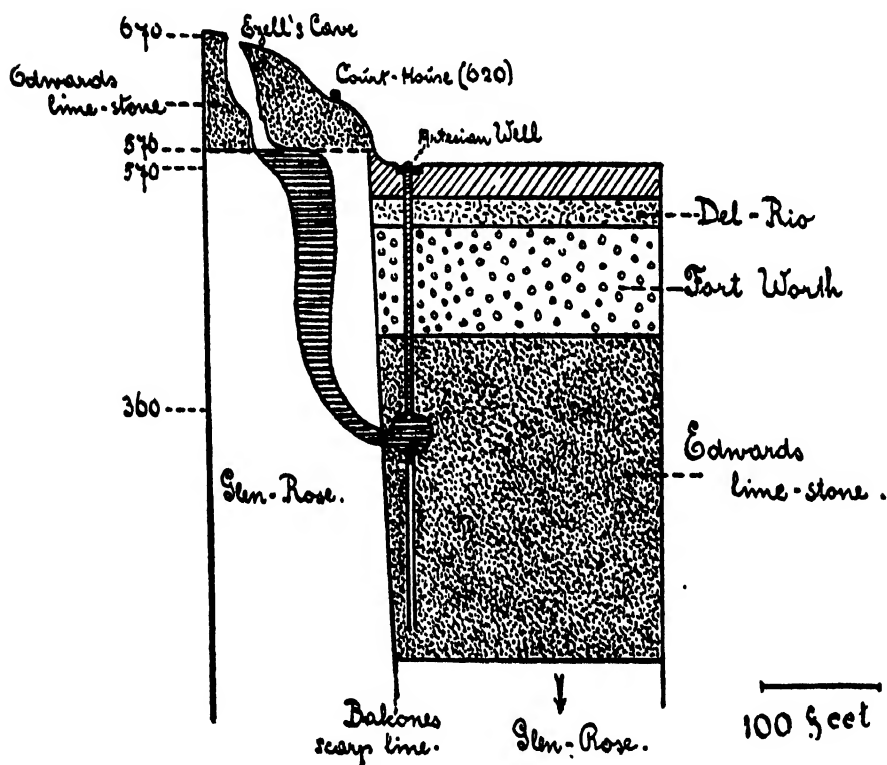
FIG. 12. Ezell's Cave. Balcones scarp line and Artesian Well of U. S. Fish Hatchery in San Marcos, Texas. Diagrammatic section showing the position of northern and southern part of the various cretaceous formations to each other after the dislocation of the Rio Grande Plain in Eocene time accomplished by faulting. The figures on the left-hand side of the diagram indicate altitude above sea level in feet.

them? And what are the reasons for the atrophy of the gland? These are problems which call urgently for investigation.

From the facts mentioned above it is certain that the *Typhlomolge* inhabit the subterranean waters which constitute the Purgatory Creek System and a subterranean water channel which supplies the San

Marcos Artesian Well. These two systems are located north and south respectively from the Balcones scarp line. On account of the faulting, though both the Purgatory Creek Caves and the Artesian Well Cave are located in the same geological formation, the latter cave occupies a position several hundred feet deeper than the Purgatory Creek Cave; this is indicated in the diagram, Fig. 12. The water in both systems is of different origin, as may be seen from this diagram. The water of the Artesian Well is the so-called "sweet water," which on the plateau, *i.e.*, in the region of the Purgatory Creek System, is carried in beds below those in which the caves of the Purgatory Creek System are located. The "sweet water" is caught by the basement beds of the Cretaceous, the Travis Peak and Glen Rose formation, where they outcrop on the plateau, and is carried down along the slanting stratum beneath the geologically higher situated Edwards limestone and towards the fault. Along the fault, however, the continuity of the water-bearing strata is broken and they come to lie in one level with the Edwards limestone of the plain; thus, here the water is forced from the Glen Rose formation into the Edwards limestone.[4]

The water of Johnson's Well, Ezell's Cave and Beaver Cave, however, is the river water of the subterranean Purgatory Creek. The level of the Purgatory Creek in these localities at present is at an altitude of about 580 feet, that of the Artesian Well 360 feet.

But as indicated above, it is quite probable that a direct communication has been established between these two systems by means of channels which according to our calculations would have a depth of about 200 feet; if this is a fact, the relation between the various bodies of water in question would be as shown in the diagram of Fig. 13.

It is of great importance to ascertain whether or not such a communication exists, since this would facilitate following the *Typhlomolge* along the course of travel and since it would permit conclusions as to the mode of the distribution of the species. Besides the suggestive structure of Ezell's Cave there are a number of facts which are in favor of the existence of a communication. If no connection between the two systems exists it would mean that the *Typhlomolge*

[4] Hill and Vaughan, page 315.

lived in the subterranean rivers before the present southern and northern parts of the Edwards limestone were separated from each other, and that after the dislocation in Eocene time part of the species was caught in the caves of the Edwards limestone of the San Marcos area south of the Balcones where it lived completely isolated from the rest of the species. Since the specimens obtained from Ezell's Cave
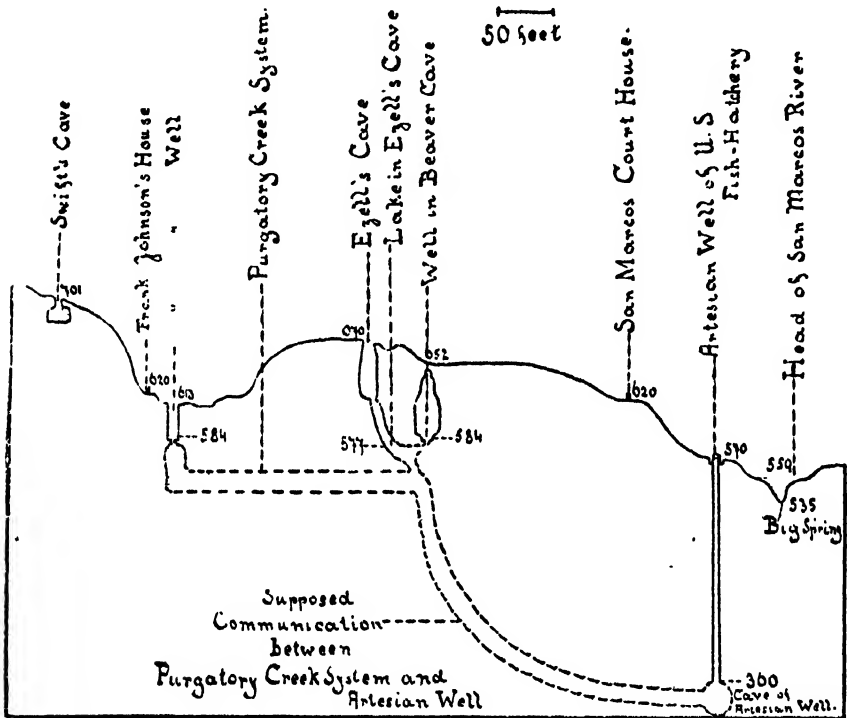
FIG. 13. Purgatory Creek System. Artesian Well and San Marcos Springs at San Marcos, Texas. Diagrammatic section reconstructed from several sections, showing the way in which these waters probably communicate with each other. The figures indicate altitude above sea in feet.

and the Artesian Well are identical, it would mean either that the species remained absolutely unchanged since ·Eocene time, or if it changed, underwent exactly similar changes in the open ponds of the subterranean Purgatory Creek and in the completely closed and water-filled subterranean caves of the Artesian Well. It is evident that none of these possibilities is probable.

Not only the Artesian Well at San Marcos but numerous other artesian wells along the Balcones escarpments are supplied from the sweet water horizon; yet from none of them, except the San Marcos Well, *Typhlomolge* has ever been reported. This would be explained if the San Marcos Well contains besides the sweet water also the Purgatory Creek water, since this certainly could not be true for the other wells. Probably the Purgatory Creek is the original habitat of the *Typhlomolge* and later on the animals migrated down to the water channels of the Artesian Well.

Also in none of the fissure springs of the Balcones scarp line, not even in the San Marcos springs though they all come from the sweet water reservoirs, *Typhlomolge* ever has been collected. The same explanation as to the artesian wells could be applied to these springs, if a communication exists between the Purgatory Creek System and San Marcos well.

Finally an incident may be mentioned here which also would speak in favor of the existence of a direct communication between the Artesian Well and the Purgatory Creek System. Mr. Mark Riley, superintendent of the U. S. Fish Hatchery, informed me that in the basin of the Artesian Well a number of catfish were kept at one time, but they disappeared gradually from the basin and it is claimed that they migrated into the tube of the artesian well. The writer is not prepared to form an opinion concerning the probability of such migration. One day, however, while I was looking for *Typhlomolge* in Ezell's Cave, I saw some fishes hiding behind the rocks. Shortly after this we caught two fishes by means of hooks which were placed near the rocks where I had seen the fishes; both were catfish. And they were the only specimens of fish which I ever saw in Ezell's Cave during the 12 days I spent there. If these were identical with the individuals kept in the basin of the Artesian Well, it certainly would be proof of the existence of a communication between the Purgatory Creek System and the San Marcos Artesian Well. It would be of great importance to trace the course of the water in Ezell's Cave and Johnson's Well down to the reservoir of the Artesian Well. As suggested by the possible migration of the catfish, such methods could be easily designed and will be employed as soon as the investigations can be continued.

In case of a connection between the two systems, the water contained in each one would be a mixture of the Purgatory Creek water and the sweet water. In all four places in question the water has the same taste and the same temperature. It contains besides the *Typhlomolge* a number of typical species, among them the *Palæmonetes antrorum*, which I found to occur in all four localities.

OTHER LOCALITIES IN PURGATORY CREEK VALLEY.

After it was found that the *Typhlomolge* inhabit subterranean regions probably representing the Purgatory Creek System, it was interesting to visit other caves of the Purgatory Creek. One of them is Swift's Cave, on the slope closing the valley towards southwest and about 1 mile above Frank Johnson's Well. The entrance to the cave is situated at an altitude of 701 feet above sea level. Though no water could be reached so far –according to what has been said above, it would have to be found about 114 feet below the entrance – there is in this cave a narrow tube leading down which has not been followed; further examination may reveal the presence of some passages to the water.

Further up the valley 14 miles above San Marcos, Boyett's Cave is located at an altitude of about 1,100 feet; there .the Purgatory Creek valley starts. No water was found in Boyett's Cave down to a depth of 50 feet and no passages leading further down were discovered. But in the large main hall of this cave one notices along the walls a whitish deposit for about $3\frac{1}{2}$ feet above the ground and forming a straight horizontal line running along the walls, as seen in Fig. 14. This indicates the former presence of water in this cave. Probably with the general disappearance of the water from the Purgatory Creek valley and its fall to deeper levels, flowing water has disappeared from the cave. There are, however, a number of small shallow pools (several inches deep) formed from dripping water in the sandy bottom of the main hall. In these pools certain crustaceans are found in large numbers, which according to Dr. Ortmann, are at least very closely related to if not identical with the species *Stygonectes flagellatus*,[5] an amphipod known from the San Marcos Well. Thus, this

[5] For further information see Benedict and Weckel.

animal, which through its mode of living is well adapted to the conditions prevailing at present in Boyett's Cave, is the only remnant there of the Purgatory Creek System fauna.
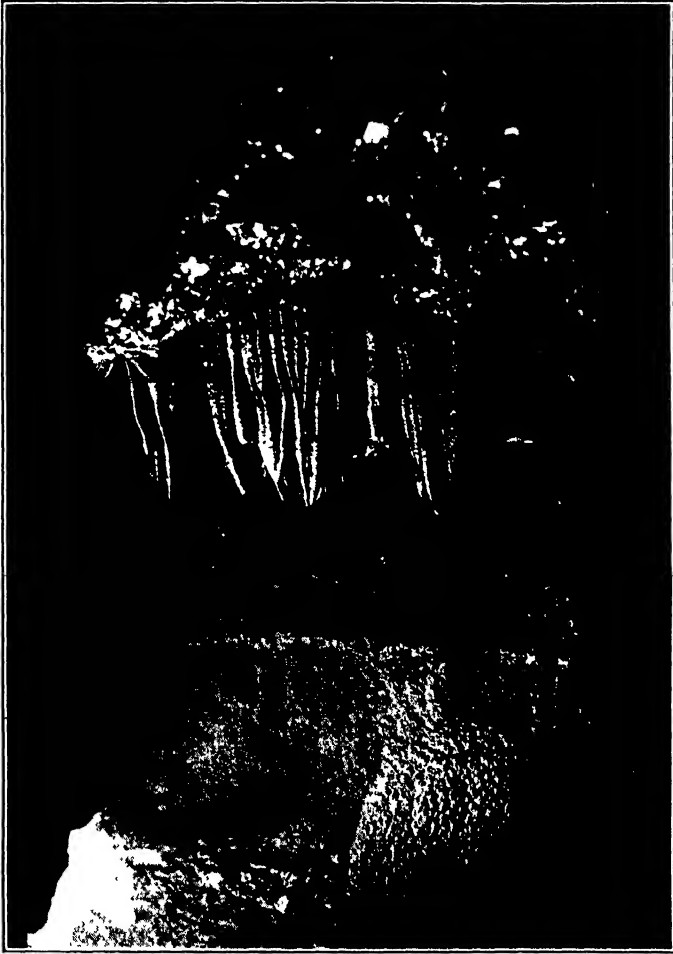
FIG. 14. Boyett's Cave.

THE SAN MARCOS SPRINGS.

According to Hill and Vaughan it is quite certain that the San Marcos Springs, like all other fissure springs along the escarpments of the Edwards Plateau, are of the same origin as the artesian wells

of this area, and hence the water of the San Marcos Springs comes from the same reservior which supplies the Artesian Well at San Marcos Fish Hatchery. We might expect therefore that between these two localities ways of communication exist along which the *Typhlomolge* may travel.

We have not so far subjected the San Marcos Springs to a thorough examination, but a brief mention may be made of certain facts valuable for future exploration. The water of the San Marcos Springs comes from funnel-like depressions of the surface (see diagram, Fig. 11), and forms a little lake which is the head of the San Marcos River. The openings through which the water emerges lie deeper (at an altitude of only 532 feet) than the surface of the water of Purgatory Creek. The surface of the lake which is artifically dammed, is 559 feet above sea level. The temperature of the water in a little spring on the bank of the lake is 21.5° C., like that of the water of the Purgatory Creek system and the Artesian Well.

Towards the south the valley at the head of the River continues and forms the bed of the San Marcos River in which the water is flowing, but this valley can be traced also north of the springs, though here it is dry.

Unconfirmed claims have been made that the "white salamander" was seen at the head of the river, but that it had developed eyes and turned brownish. These statements are, no doubt, due to the occurrence there of the larvæ of other salamanders which have been mistaken by the layman for *Typhlomolge*. But even if the animal should come up into the lake, it would be quite difficult to find it there; unaccustomed to such rapacious enemies as certain fishes which abound in the lake, the blind *Typhlomolge* would soon fall a victim.

There are, however, two localities further up in the dry valley which it might be important to examine. One is a hole resembling a well hole because of its regularity. It is about 6 feet deep. Mr. Bidler who is well acquainted with conditions as they were 20 to 30 years ago in this area, informed the writer that at one time this hole was much deeper and contained a small body of water. He assured me that one day in the water of the hole two white *Typhlomolge* were seen. At any rate this hole should be prepared for examination by removing the gravel and rocks and thus penetrating to the water, the level of

which would be approximately that of the water in Purgatory Creek. The place could be easily prepared so as to make trapping there a success.

Near this place on one of the slopes of the valley is situated another hole (on the property of Mr. Mark Riley), which much resembles the entrance to Beaver Cave. In former years, before the water which abundantly drains into that hole had washed gravel into it, one could penetrate it for some distance and reach a place at which the sound of water could be heard.

The writer believes that the exploration of the two places mentioned would lead to a definite knowledge about the presence of *Typhlomolge* in the San Marcos River valley.

OTHER PLACES TO BE EXPLORED.

It is clear that *Typhlomolge* cannot be procured in abundant number by collecting in one or two places only, since these animals pass only in small numbers from the deep water-filled caves into the open water bodies of the higher horizons. Successful collecting must have as a basis the discovery of a large number of places where some of these animals can be found. Traps must be laid in all these places and watched for some time. Assuming that from three such localities 5 specimens could be obtained in the course of two months, as was the case in Ezell's Cave, Beaver Cave and Johnson's Well, a steady and skillful worker could collect from 18 such places 180 specimens in a year, a number sufficiently large to start experimental work on the species and to keep a sufficient number for breeding stock. For this reason an attempt will be made to mention briefly a number of other places where *Typhlomolge* may possibly be found.

There are two caves on the ranch of Mr. Bender at Spring Branch, 40 miles above San Marcos and about 1,100 feet above sea level. One is a narrow channel through which the head water of Spring Branch Creek passes out. The channel is filled almost to the top with water but it is possible to penetrate it to a depth of 350 feet. Since the water is flowing quite rapidly, it is not likely to contain *Typhlo-molge*, but a more thorough search might be conducted. The other cave represents a narrow crack in the strata containing water at a depth of 45 feet. It is only a small pool, which, however, is part of

a larger body of water covered by overlapping ledges. The temperature of the water is 20.5° C. Besides frogs, some other animals inhabit this pool. They could not, however, be identified, as upon our approach they immediately dived underneath the ledge.

More important still is the water on Mr. Bremer's ranch, at the water hole of the Cypress Fork in Hays Country, a tributary branch of the Blanco River, about 1,000 feet above sea level. The water hole (Jacob's well) itself is filled with blue water which has a temperature of 22.5° C. On account of the large black basses inhabiting the hole one would not expect to find *Typhlomolge* there. But further up on one of the slopes of the dry valley is located the entrance to a cave in which the water (probably of Jacob's well) could be reached. We penetrated to a place where a number of small holes perforate the bottom of the cave; pebbles thrown into the holes evoked the sound of rather deep water. By dislocating a large rock, it would be possible to make one of the holes large enough to gain access to the water.

It might be valuable to mention a few places in which, according to Mr. S. A. Stanfield, *Typhlomolge* have been seen:

Burnet Cave, Kendall County, near Burnet.

A spring near Twin Sister Mountain, Hays County, 2 miles from .Wimberly.

A spring near Ozona, 100 miles from San Marcos.

<div align="center">SUMMARY.</div>

1. At present it is certain that *Typhlomolge rathbuni* inhabits the subterranean water of the Purgatory Creek System just north of the Balcones scarp line and one mile further up, and the caves of the Artesian Well of the U. S. Fish Hatchery at San Marcos, which seem to be in direct communication with the Purgatory Creek System by means of channels about 200 feet deep.

2. The populations of the species *Typhlomolge rathbuni* north and south of the present Balcones scarp line have not been separated from each other by the process of faulting in Eocene time, but have developed in unrestricted communication with one another.

3. No certain data are available as regards the occurrence of *Typhlomolge* in the San Marcos Springs and in the dry valley of the San

Marcos River north of the Springs.   Since the Springs come from the same water reservoir as the' Artesian Well, further investigations should be conducted.

4. All the localities containing *Typhlomolge* are located in the Edwards limestone region, but the caves of the Artesian Well are 200 feet deeper than the rest.

5. *Typhlomolge* have been found in the Purgatory Creek System at an elevation of approximately 585 feet above sea level.   Where this level could not be reached as in the upper Purgatory Creek valley, only remnants of the Purgatory Creek System fauna (*Stygonectes flagellatum*) were found.

6. The water inhabited by *Typhlomolge* seems to be slowly flowing water.

7. The temperature of the water is approximately 21.5°C. and it is inhabited by the decapod *Palæmonetes antrorum*.   Since the latter animal is much more numerous and can be detected much more easily than the *Typhlomolge*, its presence may be taken as an indication that the place is promising as regards the presence of *Typhlomolge*.

8. The rarity of the *Typhlomolge* seems to be due to the animal's habit of preferring deep lying cracks or crevices, completely filled with water at a higher pressure than exists in the more open bodies of water located at higher levels.

9. As regards the habits of the *Typhlomolge* in its natural habitat we were able to confirm Normann's observations made in the laboratory in respect to the peculiar mode of walking of this animal and its indifferent attitude to light.   But we did not find the animal particularly sensitive to water waves.

10. In feeding and swimming when aroused, *Typhlomolge* shows a close resemblance to larvæ of *Eurycea rubra*.

11. The assumption, however, that *Typhlomolge* is the larva of some unknown and still existing species of the genus *Eurycea* as made by Emmerson could not be confirmed, since with the exception of the species *Plethodon glutinosus* no tailed Batrachians were found in the caves.   More important than this assumption is the fact that *Typhlomolge*, according to Emmerson, lack a thyroid, which would explain why these animals cannot metamorphose.

12. In order to collect a large number of specimens necessary for experimental work and intensive study of the species, as many places as possible must be discovered which may contain *Typhlomolge*, and collecting must be conducted simultaneously in all these places.

13. The best method of catching the animals is by trapping, but this method must be improved. It seems probable that live bait is not attractive to the animals. Instead of relying upon bait, the large openings of the traps should be laid in the path of the animals.

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STUDIES ON X-RAY EFFECTS.

VI. EFFECT OF THE CELLULAR REACTION INDUCED BY X-RAYS ON CANCER GRAFTS.[*]

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PLATES 18 TO 20.

The theory that cancer tissue in general is more susceptible to injury by x-rays than is normal tissue has been the subject of extensive investigation.[1] As far as we have been able to determine from the literature there have been no conclusive proofs brought forward that this theory is correct. Cancer cells can undoubtedly be killed by x-rays, but judging from our experiments the amount of x-rays which can safely be given to man, without causing burns and other deleterious effects, is not sufficient to kill the cancer cell *in vitro*.[2]

In general it may be said that the x-rays have given almost uniformly beneficial results in the treatment of human cancer in only one type of malignant disease; namely, skin cancer, particularly of the basal cell epithelioma type. Many explanations have been brought forward to account for the fact that these growths are so easily affected, while other cancers, lying just beneath the skin and therefore almost as accessible to the rays, yield less uniform results. The belief among x-ray workers is that the difference depends on dosage, and their tendency has been to endeavor to increase the amount of x-rays delivered to the diseased area. Even very large doses of x-rays have failed to give uniformly good results in any but the super-

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[1] Colwell, H. A., and Russ, S., Radium, x-rays and the living cell, London, 1915, 270.

[2] Hill, E., Morton, J. J., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 89.

ficial cancers, although sometimes there is a slowing down of the progress or even a cessation of growth for a period; rarely has actual retrogression taken place.   In the vast majority of instances the treatment may be said to have had no effect, and even in the few cases in which there is improvement the benefit is only temporary.

Aside from skin cancer the only other tumors greatly affected by x-rays are the sarcomata arising from the testicle, and certain lymphoid tumors.   It is of interest to note that the tissues from which these tumors arise are the most sensitive of the normal tissues to x-rays.   Here again it is doubtful whether the malignant tissue is any more sensitive than the normal tissue from which it arose.   Some of those who question whether the cancer cell is more susceptible than normal tissue to x-rays have advanced theories to explain their results.   The two which have received most attention are (a) the idea that the dividing cell has an increased susceptibility to x-rays, and (b) the theory that the effect obtained from x-ray therapy arises from the induced obliteration of the blood vessels which diminishes the nutrition delivered to the new growth.   If the first of these explanations is correct, we should expect that the best method of treating a skin cancer would be by very frequent small doses of x-rays so as to destroy the cells as they reach the dividing stage.   Experience has shown that this method does not give the desired result.   If the obliteration of the blood vessels be the cause of the occasional retrogression, we should expect a more uniform result than is at present obtained, for blood vessel changes are quite constant.

In the past, investigators in this field have attempted to establish the efficacy of x-ray therapy by exposing tumors *in situ*.   The results viewed from the experimental side have one fallacy; namely, that they do not take into account the effect of x-rays on the animal in general and the local tissue reaction induced by this agent.   Other experimenters have exposed tumor grafts *in vitro* to x-rays and then inoculated them into animals.   In most instances when destruction of the cancer grafts resulted, the doses used were not comparable to the amount of x-rays that can safely be given to a living animal.   When no definite destructive action on the cancer grafts was noted with amounts of x-rays comparable to the dose which can safely be given to man, the view was put forward that as only one dose of

x-rays was administered in this case, while in the usual treatment of man repeated exposures are given, no conclusions can be drawn from the failure. We have recently reported a series of experiments in which the attempt was made to treat a transplantable mouse cancer *in vitro*, as nearly as possible in the same manner as that employed in the treatment of human cancers, with the exception that the dosage was magnified.[2] The cancer growths were removed at intervals of from 5 to 6 weeks, exposed to x-rays, and replanted in a new series of animals. The outcome of the experiment was at first to reduce slightly and transitorily the growth energy of the tumor, while the later treatments were without effect either on the number of takes or the rate of growth of the tumor.

Hence it may be assumed that considerable doubt still exists that x-rays in a dose suitable for a living animal, *i.e.* an amount which will not produce a burn, will exert a very great destructive action on the cancer cell. The question arises why uniformly good results should follow the treatment of skin cancer and almost as uniformly poor results be obtained in the treatment of cancers in only slightly deeper tissues. The problem involved is the immediate basis of the experiments to be described in this paper.

The studies carried out by workers in this laboratory, extending over several years, have emphasized the close relation existing between the lymphocytes and resistance or susceptibility to cancer growths.[3] They have also shown that the lymphocyte is greatly affected by x-rays, since it is possible either to stimulate by small doses the production of these cells or by larger ones practically to destroy all the lymphoid tissues of the body.[4] In looking for an explanation of the results of the treatment of human cancer with x-rays, in the light of the findings described above, we have noted two interesting observations in the literature—first, that in treating cancer of the skin the

[3] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204, 800. Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 25, 31. Murphy, Jas. B., and Nakahara, W., *J. Exp. Med.*, 1920, xxxi, 1.

[4] Taylor, H. D., Witherbee, W. D., and Murphy, Jas. B., *J. Exp. Med.*, 1919, xxix, 53. Nakahara, W., *J. Exp. Med.*, 1919, xxix, 83. Thomas, M. M., Taylor, H. D., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 75. Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1920, xxxi, 13.

method found to be best is the one in which a dose sufficient to produce a so called x-ray erythema is given, the dose not being repeated until this erythema has subsided;[5] and second, that the so called x-ray erythema, when studied histologically, consists, besides the dilation of the blood vessels, etc., of a lymphoid infiltration of the skin layers,[1] which, however, does not extend to the subcutaneous or deeper tissues. Hence, it seemed not impossible that this important difference might account for the discordant results of the treatment of cancers of the skin and of the deeper tissues. The following experiments were planned to test this point.

EXPERIMENTAL.

Intracutaneous Inoculation 7 Days after Exposure to X-Rays.—The region extending from the upper abdomen down to and including both groins was shaved carefully in healthy young mice. These animals were secured on a small board and the entire body was covered with sheet lead. An opening 15 by 20 mm. was cut in the lead so as to expose a region in the left groin extending to the midline, and this area was then exposed to x-rays in the following dose: 3 inch spark-gap, 10 milliamperes, 6 inch distance from target, and $2\frac{1}{2}$ minutes exposure. About 7 days later the skin showed a mild erythema with some scaliness. At this period small grafts of young healthy tumor from the Bashford adenocarcinoma, No. 63 series, were inoculated intracutaneously in the center of the x-rayed area and also in the corresponding position of the protected right groin. On account of the thinness of the skin, considerable caution was necessary to avoid thrusting the grafts through into the subcutaneous tissue, but occasionally this accidentally occurred. Weekly observations and measurements were made. The results of the individual experiments are shown in Table I and Text-figs. 1 and 2.

Thus it appears that there is a decided difference in the number of takes from cancer grafts inoculated into the skin of an area previously exposed to an erythema dose of x-rays, as compared to the

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[5] Knox, R., Radiography, x-ray therapeutics, and radium therapy, New York, 1916.

TABLE I.

Experiment No.	No. of animals.	Growth in x-rayed area.	Growth in protected area.
		per cent	*per cent*
1	10	40.0	90.0
2	18	33.3	94.4
3	20	40.0	100.0
4	9	44.5	100 0
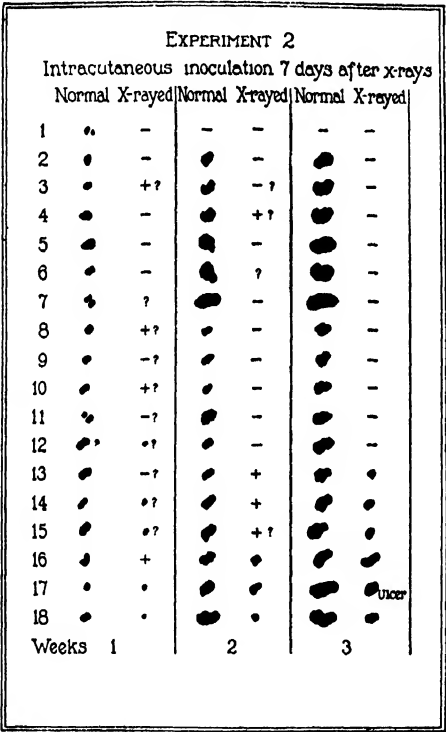
TEXT-FIG. 1. The growth of cancer grafts inoculated intracutaneously in an area 7 days after an x-ray exposure, compared to the growth of similarly inoculated grafts in an untreated area in the same animals.

number when the grafts are inoculated in the same manner into the same animal, but in an area protected from x-rays (Fig. 1). The next question to arise was whether the difference can be explained by the mechanical interference with the blood supply in the x-rayed area, from the induced changes in the blood vessels. To determine

this point a number of animals from this series were killed with ether and skinned. The skin was held before a light, by which means a clear definition of the vessels is secured. The vessels in all instances were found to be distended and numerous around the grafts in both areas, and whenever a difference was noted it was in favor of the x-rayed side. Moreover, another series of animals was injected with

TEXT-FIG. 2. A repetition of the experiment shown in Text-fig. 1.

India ink into the heart, whereby a satisfactory injection of the superficial vessels was obtained. No essential difference in the number of patent vessels on the two sides was detected.

We return now to the second possibility. If the induced cellular reaction in the skin accounts for results obtained in the treatment of human cancer, we should expect no difference in the number of takes and the rate of growth of the cancer grafts in mice inoculated under the skin rather than into the skin of an x-rayed area. The following experiments were planned to test this point.

Subcutaneous Inoculation 7 Days after Exposure to X-Rays.—A series of mice was shaved in the same manner as those described in the preceding experiments and an area on the left side of the abdomen was exposed to x-rays in the same dose, the rest of the body being completely protected. A week later a cancer graft was inoculated into the x-rayed area, but just under the skin in the loose connective tissue. Another graft was inoculated in the same manner on the opposite side of each animal, in the area protected from x-rays. The results are shown in Table II and Text-fig. 3.

TABLE II.

Experiment No.	No. of animals.	Growth in x-rayed area.	Growth in protected area.
		per cent	*per cent*
5	19	89.5	84.2
6	9	88.9	88.9
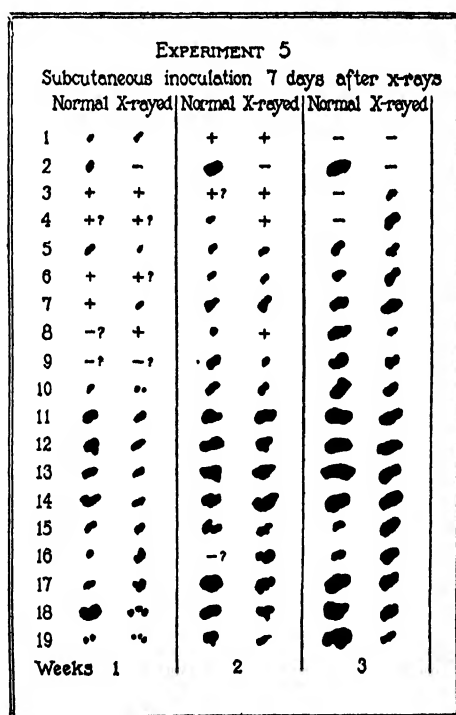
TEXT-FIG. 3. A graphic representation of the results of subcutaneous inoculations of cancer grafts into x-rayed areas as compared with the subcutaneous inoculations into untreated areas.

From the foregoing data it will be seen that the cancer graft inoculated into an x-rayed area, but under the skin instead of into the skin, grows equally as well as does a graft in a protected area inoculated in the same manner. It would seem, therefore, that whatever change is induced by x-rays which renders a tissue unsuitable for the growth of cancer is confined to the skin and does not extend even to the loose connective tissue just below it. The histological changes induced by the x-rays were next studied.

Histological Examination of Tissues of Animals Inoculated Intracutaneously after Exposure to X-Rays.—A series of mice was shaved and treated with x-rays in the same manner as in the foregoing experiments. Some of these were killed on the 3rd day and others on the 7th day after treatment. The remaining mice were inoculated intracutaneously with a graft of Bashford Tumor No. 63 in the center of the x-rayed area and in a corresponding location in the protected groin. These last animals were killed off in groups for histological study 48 hours, 4 days, and 7 days after inoculation.

The histological examination showed that 3 days after exposure to the x-rays the skin was practically normal, while 7 days after treatment a marked accumulation of lymphoid varieties of cells was present, particularly in the stratum papillare of the corium in the x-rayed area (Fig. 2), whereas the untreated area remained entirely normal (Fig. 3).

The tumor grafts in the specimens taken after inoculation were found to lie in the tela subcutanea, just beneath the stratum reticulare. In the instances in which the graft was not completely destroyed in the x-rayed area, there was a marked lymphoid reaction about it (Fig. 4), in striking contrast to the practical absence of reaction around the graft in the normal area (Fig. 5). After the destruction of the tumor graft was complete, the lymphoid infiltration in the x-rayed area of the skin was less marked.

The next experiments were made to ascertain the period of maximum effect of the changes induced in the skin by x-rays.

Exposure to X-Rays 20 Hours after Intracutaneous Inoculation.—A series of mice was shaved over the abdomen and inoculated intracutaneously in both groins as in the previous experiments. 20 hours after the tumor inoculation the graft in the left groin with an area

around it was exposed to x-rays in a dose governed by the following factors: spark-gap 3 inches, milliamperes 10, distance from target 6 inches, and time $2\frac{1}{2}$ minutes, a dose previously determined to be insufficient to destroy the cancer cell. The animals were examined weekly and measurements made of the tumors with the results shown in Table III and Text-fig. 4.

TABLE III.

Experiment No.	No. of animals.	Growth in x-rayed area.	Growth in protected area.
		per cent	*per cent*
7	7	14.4	83.4
8	11	10 0	63.7
9	20	15.0	85.0
10	18	50.0	83.4

As a control to the above observations a 3 weeks old cancer from the same series as that used for the preceding experiments was cut up into 48 small bits. These were then divided into two lots, each containing twenty-four pieces, and one lot was exposed to a dose of x-rays in the same amount as that given in the previous experiment to the area of skin in the groin. The x-rayed particles of tumor were then inoculated intracutaneously in the left groin of twenty-four mice and at the same time one of the untreated tumor particles was inoculated intracutaneously into the right groin of each mouse. At the end of 3 weeks eighteen of the twenty-four untreated grafts had produced tumors and sixteen of the twenty-four x-rayed grafts had grown. There was no appreciable difference either in the time of appearance or the rate of growth of the tumors in the two sides.

Histological Study.—Ten normal white mice were inoculated intracutaneously in both right and left groins with the strain of transplantable cancer used before. 24 hours later x-rays, governed by the same combination of factors as before, were given on the left groin over the skin area in which the cancer graft had been implanted. The right groin was left untreated for comparison. The mice were killed in groups of two, immediately, after 24 hours, 48 hours, 4 days, and 7 days after x-ray exposure.

EXPERIMENT 9
Intracutaneous inoculation
X-rayed 20 hours after inoculation

	Normal	Xrayed	Normal	Xrayed	Normal	Xrayed
1	−?	+?	−	−	−	−
2	−?	−?	−	−	−	−
3	•?	−?	−	−	−	−
4	•	−?	•	−	•	−
5	•	−	•	−	●	−
6	●	+?	•	−	●	−
7	●	+?	•	−	●	−
8	•	−?	•	−	●	−
9	●	+?	●	−?	●	−
10	•	+?	•	−	●	−
11	●	+	●	−	●	−
12	●	−	●	−	●	−
13	●	−?	●	−	●	−
14	●	+?	●	−	●	−
15	●	•?	●	−	Died	
16	●	+	●	−	●	−
17	•	−	●	−	●	−
18	●	+?	●	•?	●	•
19	●	+	●	•	●	•
20	•	•	●	•	●	●
Weeks	1		2		3	

TEXT-FIG. 4. The result of an experiment in which a cancer was inoculated intracutaneously into each groin of twenty mice and 20 hours later a dose of x-rays was given over the left groin so as to include the cancer graft and the surrounding tissue.

No detectable histological difference was found in the skin of the two sides of animals killed during the first three periods. The moderate cell infiltration about the graft in the treated, as well as the untreated side, consisted chiefly of polymorphonuclear leucocytes.

Beginning with the 4 day period an extensive lymphoid infiltration in the skin, especially about the graft on the treated side, appeared, while the graft on the untreated side was well established and attended by a moderate cell infiltration, in which polymorphonuclear cells were taking the more prominent part.

By the 7th day the tumor graft had disappeared in the treated side, but an intense lymphoid infiltration of the skin was present. In the untreated side a growing tumor was found, accompanied by some cell infiltration, although the adjacent skin showed only slight invasion.

EXPERIMENT 12

Intracutaneous inoculation 2 hours after x-rays

	Normal	X-rayed	Normal	X-rayed	Normal	X-rayed
1	•	—?	—	—	—	—
2	+	•	—	—	—	—
3	•?	+?	—	—?	—	—
4	—	+?	+?	—?	—	—
5	+	+?	—	—	—	—
6	●	—?	●	—?	●	—
7	●	÷	●	—?	●	—
8	●	—?	●	—	●	—
9	+?	+?	●	—	●	—
10	●	—?	●	—?	●	—
11	●	•	●	+?	●	—
12	•	•?	●	—?	●	—
13	●	+?	●	—?	●	—
14	•	●	●	+?	●	—
15	•	•	●	—?	●	—
16	●	+?	●	—?	●	—
17	●	•?	●	—?	●	—
18	●	●	●	+?	●	—
19	●	••	●	—?	●	—
20	•	●	●	—?	●	—
21	•?	●	●	—?	●	—
22	•?	•?	•?	●	•—	•
23	•	•	●	+?	●	•
24	•?	+?	●	+	●	•
Weeks	1		2		3	

TEXT-FIG. 5. The growth of cancer grafts inoculated intracutaneously in an area of skin 2 hours after the skin had been exposed to x-ray treatment compared to the fate of similarly inoculated grafts in an untreated area in the same animals.

While this dose of x-rays has been shown to be incapable of destroying tumor cells *in vitro*, the objection to this result as a confirmation of our first experiments is obvious, as the cancer cells in the latter experiments were exposed to the direct action of the x-rays. The following experiments were planned with the idea of avoiding this

objection and yet availing ourselves of the full time of the effect produced by the x-rays in the skin.

Intracutaneous Inoculation 2 Hours after Exposure to X-Rays.— Mice were shaved over both groins and then given the same dose of x-rays over the left groin as that given in the preceding experiments. 2 hours later intracutaneous inoculations of cancer grafts were made into the x-rayed area of the left groin and in the corresponding locality in the right groin, the latter having received no x-rays.   The results 3 weeks after these inoculations are given in Table IV and Text-fig. 5.

TABLE IV.

Experiment No.	No. of animals.	Growth in x-rayed area.	Growth in protected area.
		per cent	*per cent*
11	18	38.9	88.9
12	24	12.5	75.0

It will be seen from these last two groups of experiments that when the inoculation is made either just before or just after the administration of x-rays, the results are only slightly if any better than when the cancer inoculation is made when the reaction in the skin is at its height.

DISCUSSION AND CONCLUSIONS.

We shall not attempt to discuss the complex question of the amount of x-rays required to kill the cancer cell, for this has been dealt with extensively in recent literature.[6]   Certain facts stand out which cannot be satisfactorily explained by the direct action theory; namely, that in man skin metastases are often easily influenced by x-rays while the primary growth or even metastases in the subcutaneous tissue are resistant.   We have seen such a case in our clinic at the Hospital of The Rockefeller Institute, in which numerous skin metastases disappeared under mild doses of x-rays while metastases in the superficial glands of the neck and axilla showed no retrogression even under large doses.   The amount of x-rays delivered to the cancer cells in the latter instances was many times greater than that given to the skin metastases.   The experiments reported in this paper

[6] For a review of recent literature see Wood, F. C., and Prime, F., *J. Am. Med. Assn.*, 1920, lxxiv, 308.

offer a plausible explanation of this phenomenon. When the meta-stases or primary growth is in the skin, the x-rays induce a condition which renders it an unsuitable soil for survival of the tumor but this change does not extend as far beyond the skin layers as the subcutaneous tissue. The x-rays bring about a marked cellular reaction confined to the skin layers. It seems probable, therefore, that the explanation of the x-ray action in rendering an area unsuit-able for cancer growth is the local cellular reaction induced in this tissue. Thus this effect is brought into harmony with the observa-tions already published by us on the relation of lymphoid cell reaction and resistance to tumor growth. Certain studies recently published by Ewing[7] on the effect of radium in the treatment of human cancers indicate that a similar mechanism plays a part in the beneficial effects obtained by this mode of treatment.

Another explanation which has been proposed by many observers is that the good effect of x-rays depends on the induced blood vessel changes with a resultant deficiency of nutrition to the tumor cells. Our experiments covering this point showed that at no stage of the erythema or later during the retrogression of the tumor could any evidence of obstruction to the local blood supply be detected. In the light of the previous experiments on the relation of the lymphoid reaction to cancer immunity and the present experiments on the lymphoid cell reaction induced by x-rays and the failure of the growth of tumors in such areas, this central fact must be taken into consider-ation in accounting for the therapeutic action of x-rays in cancer. In this connection we desire to state that we do not regard the results obtained in the treatment of testicular sarcoma and certain lymphoid tumors as covered by this explanation, for like their parent tissues the cells of these respective tumors are particularly sensitive to the direct action of x-rays.

There remain to be considered the occasional beneficial results obtained with x-rays in metastatic cancer in lymph glands. This question is not a simple one. For example, in regions draining an area affected by cancer, the lymph glands often become more numer-ous and much larger than normal. Such glands are often regarded

[7] Ewing, J., *J. Am. Med. Assn.*, 1917, lxviii, 1238.

as metastatic, and yet pathologists who have examined the extirpated glands often fail to find cancer cells. These hypertrophic glands would, of course, melt away under x-rays. There is another series of events which may also give a false impression of retrogression of cancer metastases under x-rays. We have seen such an instance in the case of a large gland in the neck of a patient suffering from cancer of the breast. The nodule was exposed to vigorous x-rays and promptly retrogressed to a point where it was just palpable. The small nodule was removed at operation at this period with another involved gland which had not been subjected to the x-rays. In the latter, or untreated gland, there was a small metastasis with a fairly abundant supply of lymphoid tissue, the two making up a fair sized nodule, while in the treated gland the metastasis was found to be made up of healthy tumor cells showing no evidence of deleterious effects from the x-rays. We believe that in this case the apparent retrogression of the nodule was due simply to the destruction of the lymphoid elements of the gland with no effect whatever on the cancer cells. Finally, examples are known of definite retrogression of metastatic nodules in the superficial glands resulting from x-ray treatment to which the above explanations do not apply. Whether these are to be explained by an occasional reaction induced in the deep tissues by x-rays, or whether they represent occasional examples of tumor tissue hypersensitive to x-rays, future studies will have to determine. In view, however, of the doubt surrounding the opinion that cancers in general are more sensitive to x-rays than is normal tissue, we wish to question the advisability of the present tendency to increase greatly the dose of x-rays. We make this point since our previous studies have shown that it is possible in mice to break down the general mechanism of resistance against cancer by over-whelming doses of x-rays.[8]

SUMMARY.

Small areas of the skin in the groin of mice were subjected to an erythema dose of x-rays and a week later a cancer graft was inoculated intracutaneously into the area and at the same time a like graft was

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[8] Murphy, Jas. B., and Taylor, H. D., *J. Exp. Med.*, 1918, xxviii, 1.

inoculated in the same manner in the opposite groin protected from x-rays. The graft in the x-rayed area showed a low percentage of takes, while that in the normal skin gave the usual high percentage. When the graft was introduced into the subcutaneous tissues it grew equally as well in the x-rayed area as in the protected area.

Histological examination shows the skin layers, a few days after x-ray treatment, to be markedly infiltrated with round cells of the lymphoid type. The reaction did not extend deeper than the skin layers. It is suggested that this local lymphoid reaction induced by the x-rays controls the graft made into the skin, while its absence in deeper tissues accounts for the growth of the grafts more deeply implanted.

EXPLANATION OF PLATES.

PLATE 18.

FIG. 1. The result of an intracutaneous inoculation of cancer grafts in an area previously exposed to x-rays (left side) compared with the result of a similar inoculation in an untreated area (right side).

PLATE 19.

FIG. 2. X-rayed area of the skin of a mouse 7 days after the treatment.
FIG. 3. Untreated area of the skin of the same mouse.

PLATE 20.

FIG. 4. Cancer graft in an x-rayed area.
FIG. 5. Cancer graft in an untreated area.

FIG. 1.
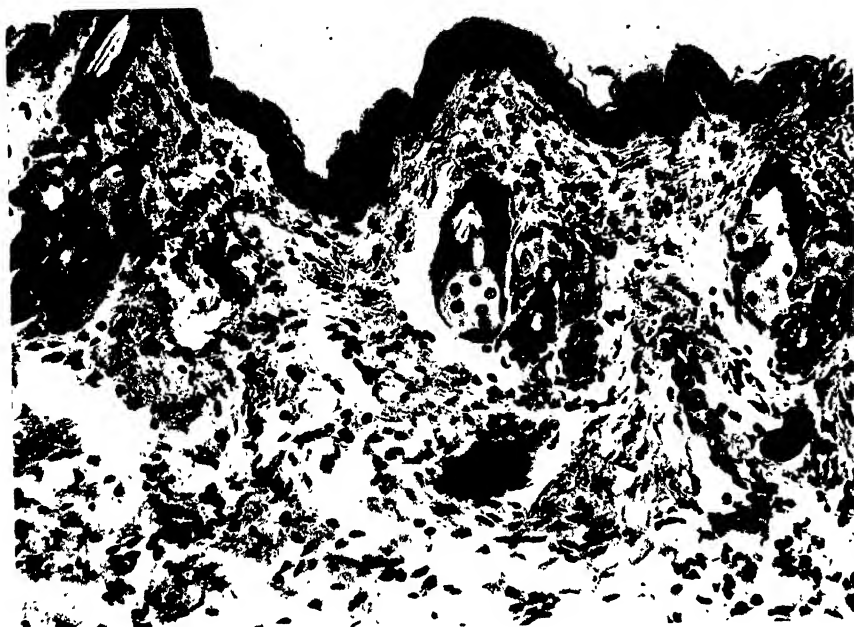
FIG. 2.
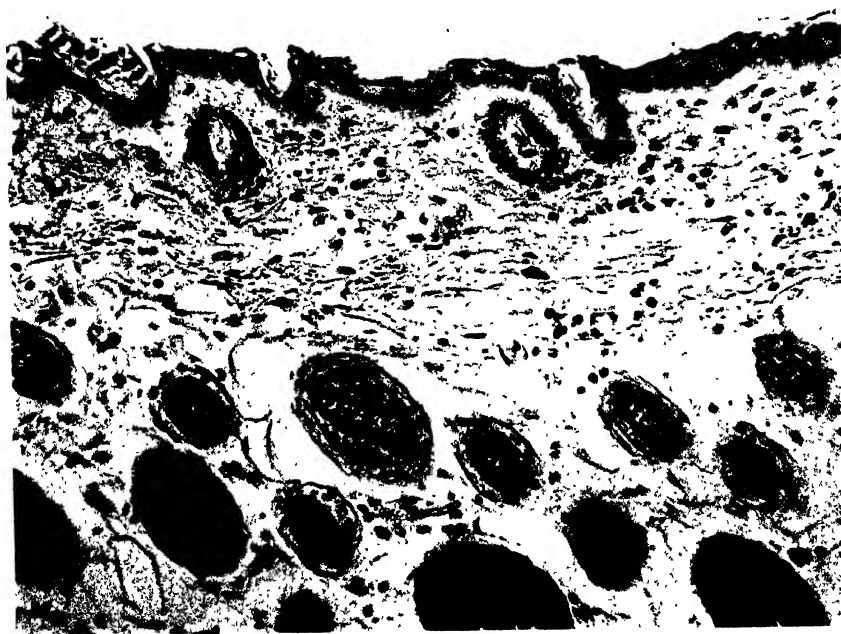
FIG. 3.

(Murphy, Hussey, Nakahara, and Sturm: X-ray effects.   VI)
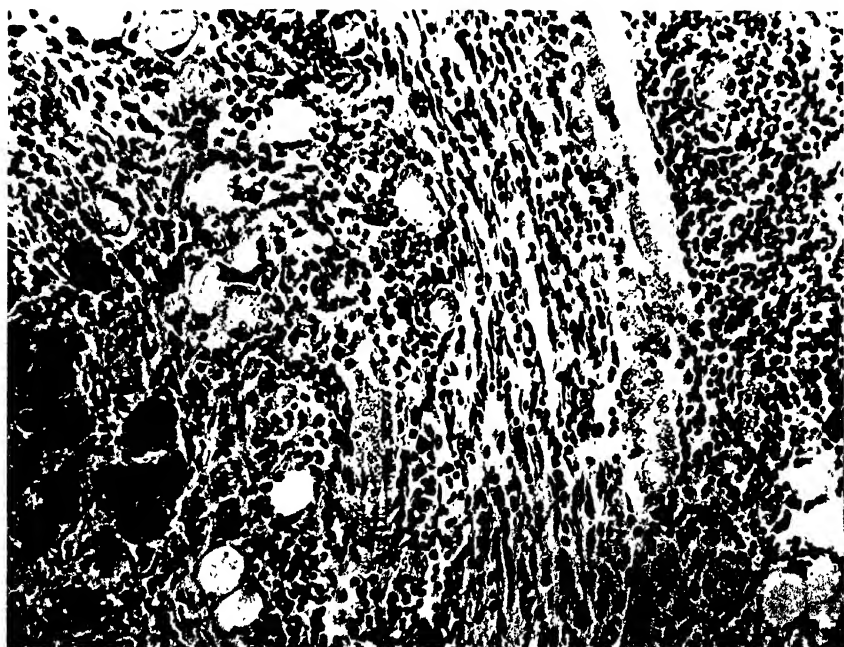
Fig. 4.
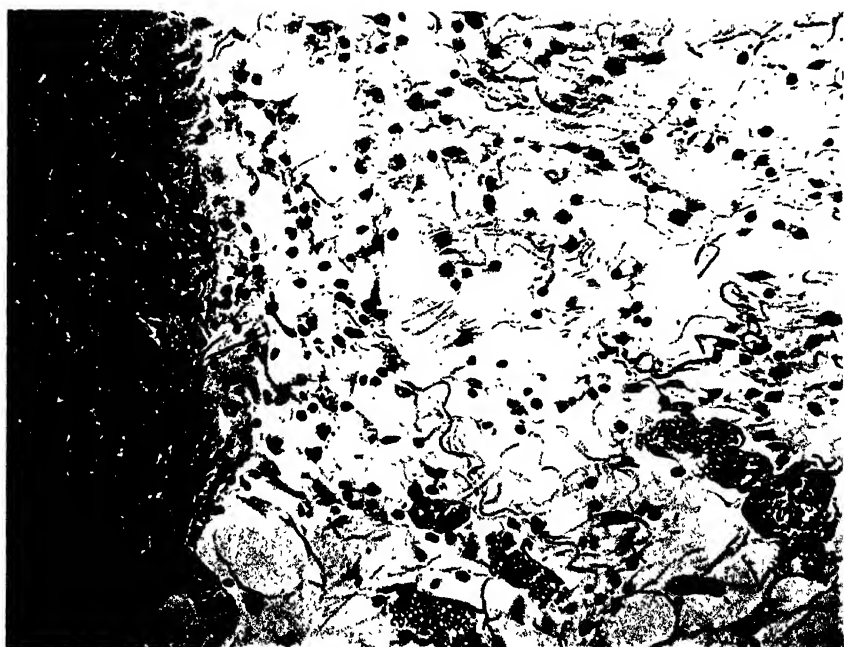
Fig. 5.

EFFECT OF INDUCED CELLULAR REACTION ON THE FATE OF CANCER GRAFTS.*

IV. STUDIES ON LYMPHOID ACTIVITY.

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PLATES 21 TO 23.

It has been shown that the induction of a general lymphocytosis is accompanied by a more or less marked immunity to cancer,[1] and that a local reaction of lymphoid cells, induced in the skin by means of x-rays, renders this tissue an unsuitable locality for the growth of cancer.[2] The reaction about a cancer graft inoculated into a mouse previously injected with homologous living tissue has a striking likeness to a local anaphylactic reaction, and is followed by a more or less complete destruction of the tumor graft. Yet in spite of this constant association of immunity and the cellular reaction, cancer investigators have been inclined to look for other explanations of the immunity phenomena.[3] If this cellular reaction is an important factor and appears to explain more or less the immunity phenomena, it should be possible to bring about a local immunity to cancer, by inducing around a graft a reaction similar to that which occurs in a generally immune animal. As the local anaphylactic reaction has

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[1] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800. Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 25, 31. Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1920, xxxi, 13.

[2] Murphy, Jas. B., Hussey, R. G., Nakahara, W., and Sturm, E., *J. Exp. Med.*, 1921, xxxiii, 299.

[3] For a review of the literature see Woglom, W. H., Studies in cancer and allied subjects. The study of experimental cancer. A review, New York, 1913.

similarities with the local effect observed about a graft in cancer-immune animals, we have tested out the influence of the former reaction on cancer grafts.

Foreign Protein Reaction in Mice.

The material most generally used to produce homologous tissue immunity in mice is defibrinated mouse blood; the amount necessary to induce a satisfactory immunity is about 0.2 cc.   In order to parallel closely this procedure the same amount of defibrinated rat blood has been used as the foreign protein in the following experiments.

0.2 cc. of defibrinated rat blood was injected into the loose connective tissues of the backs of mice, the first series of which was killed 24 hours later and the tissues prepared for and subjected to histological examination.   A considerable degree of lymphoid infiltration in the region of the injected blood (Fig. 1) was present, similar to that which occurs about an injection of defibrinated mouse blood.[4]

10 days later another series of the sensitized mice was given a second small injection of rat blood into the groin, and after another 24 hours the animals were killed and the groin tissue was prepared for histological examination.   This tissue showed a marked infiltration of lymphocytes about the injected blood, a reaction far more extensive than that occurring after a single injection and equal to that found about a cancer graft in an immune animal (Fig. 2).

Hence it is readily possible to induce a reaction to foreign protein similar to that accompanying the immunity reaction to cancer.   The following experiments were planned to test the effect of this reaction on cancer grafts.

Experiment 1.—A large normal rat was bled from the heart under aseptic precautions and the blood was defibrinated.   Of sixteen normal mice from the same strain and of about the same age, eight were injected subcutaneously with 0.2 cc. of the rat blood.   10 days later a 2½ weeks old Bashford adenocarcinoma was removed from a mouse and cut into pieces of approximately equal size.   The fragments were placed in a container and thoroughly mixed with a quantity of freshly defibrinated rat blood.   The tumor particles were then loaded into a trocar, care being taken to include a drop of the blood with each graft, and inoculated into eight mice formerly injected with rat blood and, in the same manner, into

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[4] Murphy, Jas. B., and Nakahara, W., *J. Exp. Med.*, 1920, xxxi, 1.

eight normal control mice. Measurements of the grafts were made at weekly intervals. At the end of 3 weeks, of the eight animals which had been sensitized with rat blood and afterwards inoculated with a mixture of rat blood and the cancer, three only showed tumors, while all the control mice inoculated with a mixture of rat blood and the cancer showed tumors.

Experiment 2.—The preceding experiment was repeated with ten mice in the sensitized series and ten in the control. Among the former four tumors developed, or 40 per cent of takes, while among the latter nine tumors developed, or 90 per cent of takes.

Experiment 3.—Twenty mice were given an injection of 0.2 cc. of defibrinated rat blood subcutaneously. 10 days later ten of these were inoculated with a mixture of rat blood and mouse cancer, and the other ten with mouse cancer alone. A control series of ten non-sensitized mice was also inoculated with a mixture of cancer and rat blood. The mice sensitized and inoculated with a mixture of rat blood and cancer showed four tumors, or 40 per cent of takes, the mice sensitized and injected with cancer grafts alone showed ten tumors, or 100 per cent of takes, and the control, non-sensitized mice given cancer plus blood showed nine tumors, or 90 per cent of takes.

Experiment 4.—This experiment was a repetition of Experiment 3, with ten mice in each series. The mice sensitized with rat blood and then inoculated with a mixture of rat blood and mouse tumor showed five tumors, a susceptibility of 50 per cent, the mice sensitized with rat blood and inoculated with tumor alone showed ten tumors, a susceptibility of 100 per cent, and the normal mice inoculated with a mixture of rat blood and mouse tumor showed ten tumors, a susceptibility of 100 per cent.

Experiment 5.—The experiment was again repeated with ten mice in each series. The mice sensitized with rat blood and then inoculated with a mixture of rat blood and mouse cancer showed a susceptibility of 60 per cent, while the animals sensitized and inoculated with cancer material alone were 100 per cent susceptible, as was also the normal control series inoculated with a mixture of rat blood and cancer.

Experiment 6.—This was a repetition of the preceding experiments. The results showed 50 per cent susceptibility in ten animals sensitized with rat blood and later inoculated with a mixture of rat blood and mouse cancer, 77 per cent susceptibility in a series of nine mice sensitized and inoculated with the tumor tissue alone, and 80 per cent susceptibility in the control series of ten normal control mice inoculated with the mixture of rat blood and mouse cancer.

The results obtained in the foregoing experiments are presented in Table I and Text-fig. 1.

TABLE I.

Experi- ment No.	Group A.*	Group B.	Group C.
1	62.5 per cent immunity (8 mice).		0.0 per cent immunity (8 mice).
2	60.0 per cent immunity (10 mice).		10.0 per cent immunity (10 mice).
3	60.0 per cent immunity (10 mice).	0.0 per cent immunity (10 mice).	10.0 per cent immunity (10 mice).
4	50.0 per cent immunity (10 mice).	0.0 per cent immunity (10 mice).	0.0 per cent immunity (10 mice).
5	40.0 per cent immunity (10 mice).	0.0 per cent immunity (10 mice).	0.0 per cent immunity (10 mice).
6	50.0 per cent immunity (10 mice).	23.0 per cent immunity (9 mice).	20.0 per cent immunity (10 mice).

* Group A was composed of animals sensitized with 0.2 cc. of rat blood and 10 days later inoculated with a mixture of rat blood and mouse cancer. Group B was made up of mice sensitized with 0.2 cc. of rat blood and 10 days later inoculated with mouse cancer alone. Group C was made up of mice not sensitized but inoculated with a mixture of rat blood and mouse cancer.

Histological Study of the Fate of the Cancer-Rat Blood Mixture Inoculated into a Sensitized Animal.

It has been shown that the reaction which takes place about the immunizing injection of mouse blood into a mouse is similar to that about an injection of rat blood in a mouse. The reaction which takes place around a cancer graft in an immunized mouse has been shown to be similar to that which occurs around injected rat blood in a mouse previously sensitized to rat blood. The following experiment was undertaken to supply material for the histological study of the fate of a cancer graft mixed with rat blood when inoculated into a mouse previously sensitized to rat blood.

A series of mice was injected with 0.2 cc. of rat blood and 10 days later inoculated with a mixture of rat blood and mouse tumor. These mice were killed in groups at 24 hour intervals up to the 7th day and histological studies made of the grafts. 24 hours after inoculation there was a massive lymphoid and a mild polymorphonuclear reaction about the graft (Figs. 3 and 4). The reaction was still marked on the

Sensitized to rat blood. Inoc. with mixture of rat blood and mouse cancer	Sensitized to rat blood. Inoc. with mouse cancer	Not sensitized Inoc. with mixture of rat blood and mouse cancer
60.0% immune.	Experiment 2.	10.0% immune.
60.0% immune.	Experiment 3 0 0% immune.	100% immune
50 0% immune.	Experiment 4. 0 0% immune.	0 0% immune.
Weeks 1 2 3	1 2 3	1 2 3

TEXT-FIG. 1. The rate of growth of mouse cancer inoculated in a mixture with rat blood into mice previously sensitized to rat blood, compared to the rate of growth when the cancer alone is inoculated into sensitized mice and the rate of growth when the cancer and rat blood are inoculated into non-sensitized mice.

2nd day, but by the 3rd day it had begun to diminish and the graft was more or less completely destroyed. After this period there was a rapid subsidence of the reaction. The reaction described above is similar to that seen around the cancer graft in an immunized animal.

Desensitizing Effect of Generalized Doses of X-Rays.

In a previously reported experiment it was shown that mice rendered potentially immune by the injection of mouse blood could be reduced to a susceptible state by properly regulated exposure to x-rays, administered between the time of the immunizing dose and that of the cancer inoculation.[5] It has likewise been shown that x-rays administered at certain periods after a foreign protein injection desensitize an animal to such an extent that no anaphylactic shock results after a second injection of the protein.[6] These facts have led us to test the effect of x-rays on the immunity resulting from sensitization of mice with rat blood and the subsequent inoculation of mouse cancer mixed with rat blood.

Normal white mice were injected subcutaneously with 0.2 cc. of defibrinated rat blood. These mice and another group of mice which had not been sensitized were then given daily doses of x-rays for 8 days. The dose was governed by the following factors: spark-gap $2\frac{1}{4}$ inches, milliamperes 10, time 2 minutes, and distance from target 12 inches. 10 days after the sensitizing injection these animals and several normal controls were inoculated with a Bashford adenocarcinoma mixed with defibrinated rat blood. The results from these experiments are given in Table II and Text-fig. 2.

TABLE II.

Experiment No.	Group A.*	Group B.	Group C.
7	10.0 per cent immunity.		20.0 per cent immunity.
8	27.2 " " "	10.0 per cent immunity.	20.0 " " "

* Group A comprises thirty-two mice sensitized to rat blood, which were given eight exposures to x-rays and were then inoculated with a mixture of rat blood and mouse tumor. Group B was made up of ten mice not sensitized but given the same amount of x-rays as Group A and then inoculated with cancer alone. Group C was composed of twenty normal animals inoculated with a mixture of rat blood and mouse cancer, which had received no previous injection of blood and no x-rays. The total number of mice used in these experiments was 62.

[5] Murphy, Jas. B., and Taylor, H. D., *J. Exp. Med.*, 1918, xxviii, 1.
[6] Hussey, R. G., and Murphy, Jas. B., unpublished observation.

TEXT-FIG. 2. The effect of generalized x-rays on the immunity to transplanted cancer resulting from a local anaphylactic reaction.

Effect on the Foreign Protein Reaction of X-Rays Administered Locally.

The foregoing experiments show that mice develop an enhanced refractory state to the growth of transplanted tumor if they are first sensitized with rat blood and inoculated 10 days later with a mixture of rat blood and mouse tumor. Histological examination made at intervals after the tumor inoculation shows that there is a definite local reaction, made up principally of cells of the lymphoid series, which takes place around the tumor cells. This reaction reaches its maximum about 24 hours after inoculation and then gradually sub-

sides. It seems reasonable in the light of previous observations that the cellular elements constituting the reaction play some active part in the mechanism bringing about the refractory state.

On the assumption that the latter statement is true, it would seem to follow that if it were possible to destroy these cells and at the same time not to injure the tumor cells, the refractory state potentially present might be inhibited to some measure. Since it has been shown that the x-rays in moderate amounts have little if any direct effect on the viability of the cells of transplanted tumor when exposures are made directly to the tumor,[7] and since it is well known that the x-rays when properly regulated have a selective destructive action on the lymphoid elements of the body,[8] it was thought possible through these means to effect the purpose stated above.

Normal mice were inoculated subcutaneously with 0.2 cc. of defibrinated rat blood and 10 days later inoculated in the groin with a bit of Bashford adenocarcinoma mixed with defibrinated rat blood. 15 to 20 hours after the inoculation the mice were covered with sheet lead in which an aperture had been made large enough to allow the rays to affect the area around the graft. This area was then exposed to the x-rays in the following dose: spark-gap 8 inches, milliamperes 5, time 4 minutes and 38 seconds, distance from target 8 inches, filtered through 3 mm. of aluminum. For controls to the above experiment, normal mice were inoculated with bits of the same tumor and were exposed to the same amount of x-rays, and other normal mice were inoculated with tumor and rat blood without previous sensitization or after-treatment with x-rays. The results are shown in Table III and Text-fig. 3.

TABLE III.

Experiment No.	Group A.*	Group B.	Group C.
9	30.0 per cent immunity.		20.0 per cent immunity.
10	38.0 " " "	10.0 per cent immunity.	20.0 " " "

* Group A was made up of thirty-one mice, sensitized to rat blood, which were

[7] Hill, E., Morton, J. J., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 89.

[8] Heineke, H., *Mitt. Grenzgeb. Med. u. Chir.*, 1904–05, xiv, 21.

inoculated after 10 days with a mixture of rat blood and mouse cancer; 20 hours later they were given a local dose of x-rays. Group B was composed of normal mice inoculated with a mouse cancer and 20 hours later given a local dose of x-rays. Group C consisted of twenty normal mice inoculated with a mixture of mouse tumor and rat blood.

			Experiment 10.					
	Sensitized. Tumor and blood inoc. X-rayed locally 20 hrs. later. 38.0% immune.			Normal. Tumor (alone) inoc. X-rayed locally 20 hrs. later. 10.0% immune.			Normal. Tumor and blood inoc. 20.0% immune.	
1	+	−	−	+	−	−	+	−	−
2	−	−	−				?	−	−
3	+	−	−						
4	−?	−	−	+					
5	−?	−	−						Died
6	+?	−	−						
7	−?	−	−						
8	+?		−						
9									
10	+								
11									
12									
13									
14									
15									
16									
17									
18									
19									
20									
21									
Weeks	1	2	3	1	2	3	1	2	3
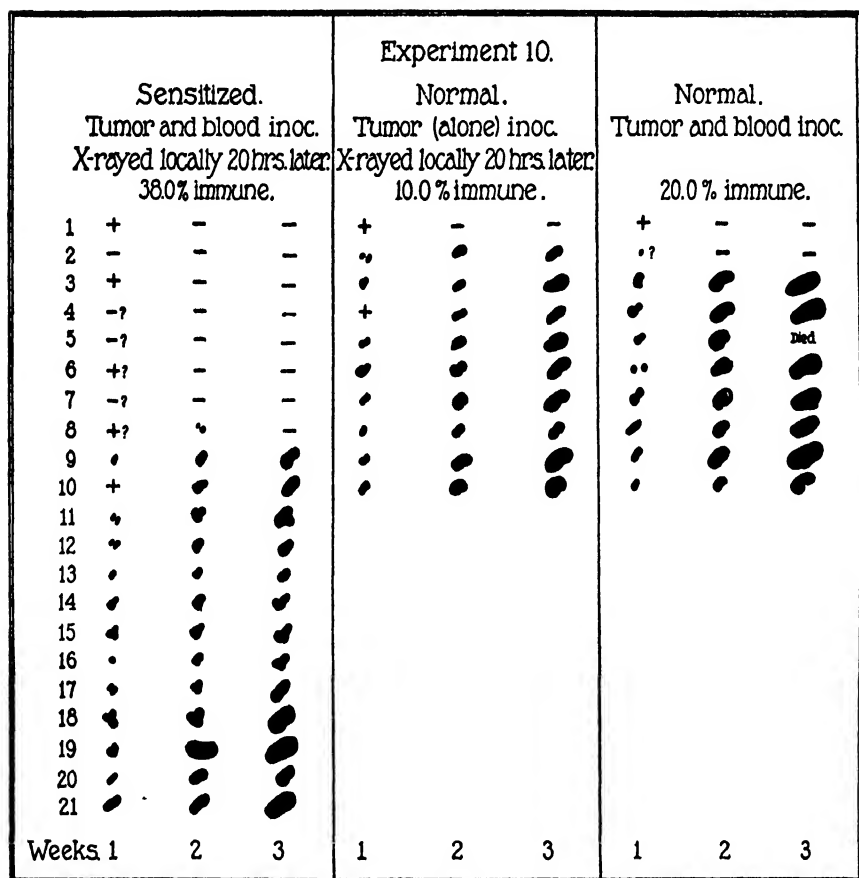
TEXT-FIG. 3. The effect of x-rays on the immunity to transplanted cancer resulting from a local anaphylactic reaction when the x-rays are administered locally over the cancer graft 20 hours after inoculation.

Histological Study.—Twelve normal white mice were inoculated with 0.2 cc. of rat blood, and 10 days later inoculated with rat blood plus a fragment of a Bashford Adenocarcinoma No. 63. 24 hours after the second inoculation a dose of x-rays was given locally over

the area of the cancer implantation. Two of the mice were killed just before the x-rays were given, and two others immediately afterwards. Eight mice were killed, in groups of two, 24 hours, and 3, 6, and 10 days after x-ray treatment.

24 hours after the second inoculation an extensive cell infiltration was found in the area of the subcutaneous tissue where the mixture of heterologous blood and homologous cancer tissue had been inoculated (Fig. 5). The cells participating in the reaction were chiefly of the lymphoid variety, the polymorphonuclear cells being less numerous.

In the specimens taken immediately after the x-rays were given there was a striking reduction of these cells (Fig. 6) in the area which had been thickly infiltrated immediately before the x-ray treatment. How this reduction of the cells is brought about is a matter of conjecture, but it should be mentioned that no necrotic cells were found in the x-rayed area. 24 hours after the x-ray treatment and later the cell infiltration was gradually restored, although it did not become so extensive as it was before.

The temporary suppression of the lymphoid reaction effected by the local dose of x-rays seems to indicate that the removal of these cells is one of the factors which plays a part in allowing the graft to grow in the sensitized animal.

From the two foregoing groups of experiments the conclusion is drawn that it is possible to overcome, to a certain extent, the immunity to cancer resulting from a local anaphylactic reaction, in two ways. In either case we consider that the effect follows the prevention of the local cellular infiltration from taking place or from becoming effective. In the first of these two experiments animals were desensitized so that the second injection of foreign protein did not call forth the local cellular reaction. In the second experiment the lymphocytes taking part in the local reaction were destroyed by x-rays before they had time materially to affect the cancer graft.

DISCUSSION.

The foregoing experiments offer further evidence of the hypothesis that the so called immunity to the transplanted cancers of mice depends on a local cellular reaction in which cells of the lymphoid

type play the principal part. The usual method of producing this immunity is through the injection of a quantity of living homologous tissue, which leads to a non-specific immunity, which in turn is directed against a great variety of cancers and sarcomas, as well as against transplanted normal tissue. It has been suggested that this immunity phenomenon is analogous to the so called anaphylactic reaction, but the exact nature of the relation had never been demonstrated. The experiments reported here indicate that this relation is quite close. The first injection prepares or sensitizes, and the second injection of the cancer cells calls out a cellular exudate such as is observed in local anaphylactic reactions. Why living cells are necessary for the sensitizing dose is not evident, unless it requires living cells to sensitize to living cells. The fact is unmistakable, from the experiments reported here, that the condition of local anaphylaxis renders the tissues affected unsuitable for the growth of a cancer graft, and the histological changes which arise correspond with those seen about a cancer graft in an animal immunized by a previous injection of homologous tissue. That the cellular exudate is the essential inhibiting agent is indicated by the fact that when this exudate is prevented from arising or is arrested, the protective effect is either annulled or materially reduced. It is still uncertain whether the desensitization induced by x-ray exposure results in such a general destruction of the lymphocytes that the number left is insufficient to yield the local reaction, or whether it depends on some other factor. Reasoning from the observed fact that x-rays are capable of actual desensitization even to the extent of preventing anaphylactic shock from a second injection of foreign protein, one may well consider whether the failure of immunity under these conditions does not arise from the inability of the desensitized animals to call out the usual cellular exudate. Inasmuch as the local destruction of the cellular exudate is sufficient to annul or reduce the immunity, it is unlikely that it is of the nature of a serum-carried resistance, for the amount of x-rays needed for this purpose is so small and its area of application so limited that it could hardly produce a general effect. In brief, there seems to be no other explanation for the results recorded than that cells of the lymphoid type are capable of preventing the growth of a transplanted cancer when present locally in sufficient

numbers. Hence, we conclude that these cells are an active agent in bringing about the so called immunity condition to transplanted cancer.

SUMMARY.

Mice sensitized by an injection of 0.2 cc. of rat blood and 10 days later inoculated with a mixture of rat blood and a transplantable mouse cancer showed a high degree of immunity to the cancer growth, while mice sensitized in the same manner and inoculated with cancer graft with no rat blood showed no immunity. Likewise, non-sensitized mice inoculated with a mixture of rat blood and cancer cells showed no immunity.

Mice sensitized to rat blood and then given a series of doses of x-rays between the time of this injection and the inoculation of the cancer-rat blood mixture showed a suppression of the factors affording protection or immunity, since the cancers grew as well in these animals as in the controls.

Mice were sensitized with rat blood and 10 days later inoculated with a cancer-rat blood mixture. 20 hours after the inoculation when the cellular exudation was at its height, the cells were destroyed by a local dose of x-rays. The degree of immunity was reduced and the cancers grew almost as well as in the controls.

EXPLANATION OF PLATES.

PLATE 21.

FIG. 1. Subcutaneous tissue of a mouse inoculated with rat blood, 24 hours after the inoculation.

FIG. 2. Subcutaneous tissue of a mouse inoculated with rat blood, 24 hours after a second inoculation of rat blood.

PLATE 22.

FIG. 3. Subcutaneous tissue of a mouse sensitized to rat blood, 24 hours after an inoculation with rat blood mixed with mouse tumor.

FIG. 4. The same as Fig. 3, but in higher magnification.

PLATE 23.

FIG. 5. Subcutaneous tissue of a sensitized mouse, 24 hours after an inoculation of a cancer-rat blood mixture, showing an extensive cell infiltration.

FIG. 6. Subcutaneous tissue of a mouse which was sensitized with rat blood, then inoculated with a mouse cancer-rat blood mixture, and 24 hours later given a local dose of x-rays. The tissue was removed immediately after the treatment.
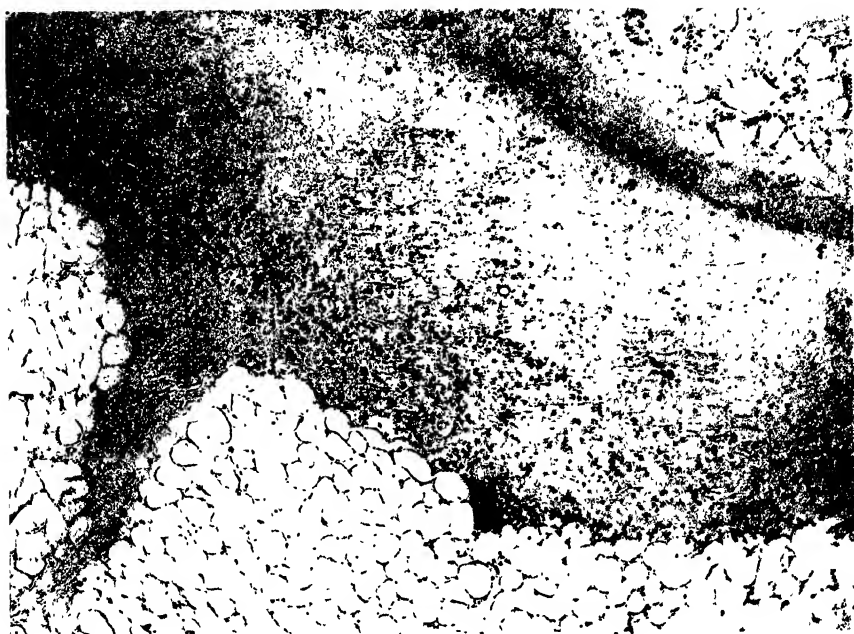
Fig. 1.
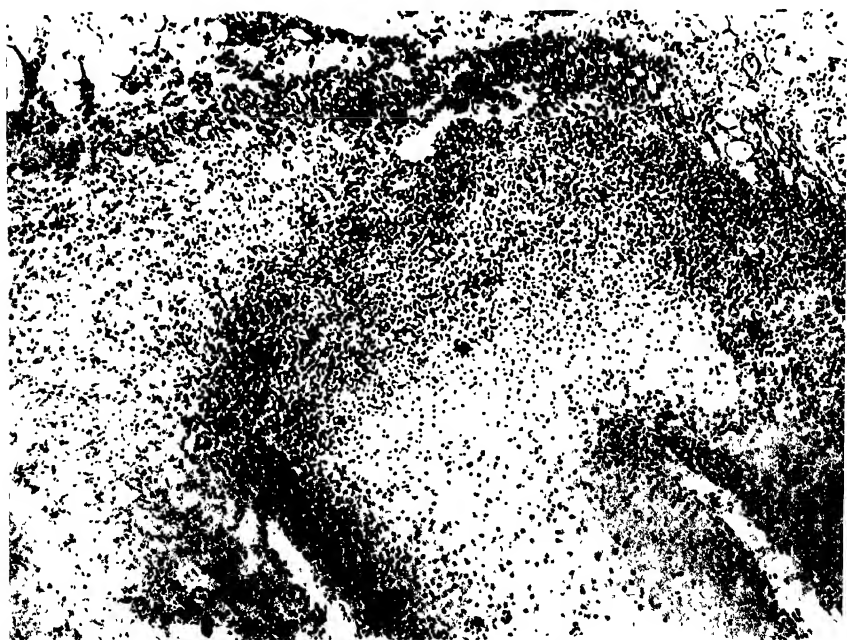
Fig. 2.

(Murphy, Hussey, Sturm, and Nakahara: Cancer grafts.   IV.)

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

THE LYMPHOCYTE IN NATURAL AND INDUCED RESIST-
ANCE TO TRANSPLANTED CANCER.

VI. Histological Comparison of the Lymphoid Tissue of
Naturally Immune and Susceptible Mice.*

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 24 to 26.

It has long been known that if a number of normal white mice are
inoculated with fragments of transplantable mouse cancer, certain
of them will, as a rule, prove to be naturally refractory. The striking
histological difference in the reaction about cancer grafts in naturally
resistant and in susceptible animals is too well known to be discussed.
Murphy and Morton[1] showed that this resistant state is accompanied
by a marked lymphocytosis in the blood, absent in the susceptible
animals, and also that treatment of normal animals with x-rays
destructive to lymphocytes removes at the same time the immunity
otherwise present. Hence, the conclusion is drawn that the lympho-
cyte is a factor in the state of natural immunity.

Histological studies[2-5] paralleling our observations on the circu-
lating lymphocytes[1, 6, 7] indicate that the lymphoid tissue is the basis
of the immunity to transplanted cancer induced by intense dry heat,

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[1] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204.

[2] Nakahara, W., *J. Exp. Med.*, 1919, xxix, 17.

[3] Nakahara, W., *J. Exp. Med.*, 1919, xxix, 83.

[4] Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1920, xxxi, 13.

[5] Murphy, Jas. B., and Nakahara, W., *J. Exp. Med.*, 1920, xxxi, 1.

[6] Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 1.

[7] Thomas, M. M., Taylor, H. D., and Witherbee, W. D., *J. Exp. Med.*, 1919,
xxix, 75.

and of that induced by small doses of low penetrating x-rays and by the injection of homologous living cells.   Hence, it would appear that a histological comparison of lymphoid organs of naturally immune and naturally susceptible mice to transplanted cancer might give enlightening results, and it was with this end in view that the present study was undertaken.

Material and Method.

Preliminary experiments indicated that more or less definite histological changes take place in lymphoid organs, especially in the spleen, soon after cancer inoculation.   These changes are not extensive, but are, nevertheless, of a characteristic nature; hence it is not difficult to determine at the end of the 3rd week of the experiment whether a given spleen comes from an immune or a susceptible mouse.   Therefore, we studied the lymphoid organs of young white adult mice inoculated with a given strain of tumor (Bashford Adenocarcinoma No. 63) and killed 3 weeks later.   The tissues were fixed in Carnoy's 6–3–1 and stained with eosin-methylene blue, Ehrlich's hematoxylin and eosin, or Heidenhain's iron-hematoxylin.   The material consisted of the spleen and lymph nodes from 105 mice, of which 29 came from mice immune to the inoculated cancer, and the remaining 76 from animals with tumors.

OBSERVATIONS.

The results of histological examination of the lymphoid organs of the naturally immune and susceptible mice are brought out in Tables I and II.   It will be noted that the distinction between these two groups is not clear-cut, but that a complete series of intergradations in the changes exists.   However, the two groups differ decidedly in the general tendency of the changes, as is brought out in Table III. Descriptions of the nature of the typical changes in lymphoid organs of immune, as contrasted with those of susceptible mice, are given below.

Spleen.

Immune Mice.—The general histological appearance of a typically immune spleen is quite characteristic (Fig. 1). The Malpighian bodies are enlarged and show practically no pycnotic cells. Phagocytic cells containing cell fragments found in the germ centers are few, while mitotic figures present in these areas are striking in number (Fig. 2). The superabundance of mitotic figures may be taken to indicate a hyperactivity in tissue proliferation. In typical examples the splenic pulp contains a large quantity of lymphoid tissue which is especially abundant around the vessels. The lymphoid tissue in the pulp contains a considerable number of mitotic figures, which occur only sparingly in normal splenic pulp. Comparatively few degenerating cells with pycnotic or fragmental nuclei are found. The pulp space contains a very small amount of blood, and the deposits of pigment are inconspicuous.

Susceptible Mice.—The spleen of a typically susceptible animal presents a different histological picture (Fig. 3). The Malpighian bodies are small and contain very few mitotic figures, while the phagocytic cells with ingested cell fragments are abundant. A few necrotic cells may be found in the peripheral portion of the Malpighian body. The lymphoid tissue of the splenic pulp is very small in amount, and certain vessels may be entirely devoid of this type of tissue. Some of the small perivascular accumulations of lymphoid cells are partly pycnotic. The deposits of pigment are pronounced (Fig. 4), and there is a large quantity of the blood which fills up the spaces unoccupied by lymphoid tissue.

Lymph Nodes.

Immune Mice.—Mesenteric, inguinal, and often also cervical and axillary lymph nodes were studied. The changes in these nodes are entirely parallel to those found in the spleen, although they are not so striking. In typical examples of immune mice the lymphoid tissue, in the cortex as well as in the medulla, contains many mitotic figures, while there are scarcely any necrotic cells. The pulp spaces are clouded by a large number of lymphocytes (Fig. 5), while other types of cells, such as those of the endothelial group, are not much in evidence.

Susceptible Mice.—Compared to the condition of lymph nodes in immune animals, the lymphoid tissue in various nodes in typically susceptible animals shows a striking inhibition of proliferative activity. This may be judged from the scarcity of mitotic figures in the tissue. Some of the cells in the lymph cords are necrotic. Only a few lymphocytes are found in the pulp spaces, the latter being occupied to a great extent by proliferating endothelial cells (Fig. 6).

In addition to the more typical changes already described, it was noted in several instances that plasma cells appeared in large number in the lymph cords. In fact, in such cases, portions of the cords were almost entirely made up of these cells. This change, however, did not appear to be related to the immunity or susceptibility of the animals, as it occurred irregularly. It may also be mentioned that a few susceptible animals showed a considerable number of polymorphonuclear leucocytes in the lymph cords and even in the cortex, and mast cells in the peripheral sinuses.

The descriptions above refer to the extreme types. As might be expected, almost every gradation is encountered between the extremes, for it is well known that various degrees of immunity exist. In the highly resistant animals the tumor grafts are quickly destroyed, while in others only after a period of growth is the cancer overcome. The highly susceptible animals offer little or no inhibitory effect, and the grafted tumor grows at a rate limited only by its growth energy and the ability of the host to supply stroma, while the less susceptible animals show evidences of a definite retarding effect on the rate of growth of the graft.

In order to show the degrees and variations in the points of difference between the histological appearance of the lymphoid tissue of the susceptible and the resistant animals, we have presented in Tables I and II the results of the study of the individual mice. For the spleen we have included the following points: (*a*) the relative size of the nodules, (*b*) the amount of lymphoid tissue in the pulp, (*c*) the number of mitotic figures present, (*d*) the amount of necrosis of the cells, and (*e*) the amount of blood and pigment present. For the lymph nodes we have indicated (*a*) the number of mitotic figures, (*b*) the degree of necrosis, and (*c*) the number of lymphocytes and endothelial cells present in the pulp. The material from the indi-

vidual animals was studied and the records were made without any knowledge of whether the tissues came from a susceptible or immune mouse.

TABLE I.

Immune Mice.

Immune Mouse No.	Spleen.						Lymph nodes.			
	Size of nodules.	Lymphoid tissue in pulp.	Mitosis in lymphoid tissue.	Necrosis in lymphoid tissue.	Blood.	Pigment.	Mitosis in lymphoid tissue.	Necrosis in lymphoid tissue.	Lymphocytes in pulp spaces.	Endothelial cells in pulp spaces.
1	±*	+	±	−	±	±	−	−	±	±
2	−	±	−	−	±	+	±	−	±	−
3	±	−	±	−	−	±	−	±	+	±
4	+	+	+	−	−	±	−	−	±	±
5	−	±	−	±	+	−	−	±	+	±
6	+	+	+	±	−	−	±	±	±	+
7	±	+	−	±	±	±	±	−	+	±
8	+	+	+	±	−	−	−	−	±	±
9	+	+	±	−	±	±	+	±	±	±
10	+	±	±	−	±	−	±	−	±	−
11	+	+	+	−	−	±	±	−	±	−
12	+	+	±	±	−	±	±	−	±	−
13	+	+	−	−	±	−	+	−	+	−
14	+	+	±	+	±	−	+	−	+	±
15	+	+	±	±	±	±	+	−	±	−
16	+	±	+	±	±	−	+	±	+	−
17	±	±	±	+	±	±	±	−	±	+
18	±	±	±	±	+	±	−	±	−	±
19	+	±	±	+	±	±	±	−	±	±
20	+	±	±	−	−	±	±	±	+	±
21	+	+	+	±	±	−	±	−	±	±
22	+	±	±	±	±	±	±	±	±	+
23	+	±	+	±	±	±	+	−	+	±
24	±	±	±	±	±	±	±	±	+	±
25	+	±	±	−	−	±	±	±	+	±
26	+	±	+	±	±	−	±	±	+	±
27	+	±	±	±	−	−	±	−	+	−
28	±	±	±	±	±	−	±	±	+	±
29	+	±	−	±	−	−	±	−	±	±

* In the tables + indicates an increase above the normal; ±, approximately normal; −, a decrease below the normal.

TABLE II.

Susceptible Mice.

Susceptible Mouse No	Spleen.						Lymph nodes.			
	Size of nodules.	Lymphoid tissue in pulp.	Mitosis in lymphoid tissue.	Necrosis in lymphoid tissue.	Blood.	Pigment.	Mitosis in lymphoid tissue.	Necrosis in lymphoid tissue.	Lymphocytes in pulp spaces.	Endothelial cells in pulp spaces.
1	±	±	−	−	±	+	−	−	±	+
2	−	±	±	−	−	±	−	−	±	±
3	−	−	−	±	+	+	−	−	±	+
4	−	±	−	±	±	±	−	−	±	±
5	±	−	−	±	+	+	−	±	±	±
6	−	−	−	−	±	+	−	±	±	−
7	−	±	−	−	±	±	−	−	−	±
8	−	−	±	−	−	−	−	+	±	±
9	−	±	±	±	−	±	−	+	±	±
10	−	−	±	±	−	±	−	+	−	−
11	−	±	−	±	±	±	−	+	±	−
12	±	+	±	−	±	−	±	+	±	+
13	+	+	+	±	−	−	−	−	±	+
14	−	−	−	±	+	−	−	+	−	+
15	±	−	−	±	+	±	−	±	−	±
16	−	±	−	−	−	+	−	±	+	±
17	±	±	±	−	−	−	−	+	±	+
18	±	±	±	−	−	±	−	+	+	±
19	−	−	−	+	+	±	−	+	±	+
20	−	−	−	+	+	+	−	±	±	+
21	−	±	−	+	±	−	±	±	±	±
22	±	±	−	±	±	±	−	−	±	±
23	±	±	±	±	−	±	−	±	−	±
24	±	−	−	±	+	±	±	±	±	±
25	−	±	−	±	±	±	−	±	±	±
26	−	−	−	+	+	−	−	−	±	+
27	−	±	±	+	±	−	±	−	±	±
28	±	+	±	±	±	±	±	+	±	+
29	−	±	−	+	±	±	−	±	−	+
30	−	−	−	+	+	±	±	+	±	+
31	−	−	−	±	±	±	−	±	−	+
32	−	−	−	−	±	±	−	±	±	±
33	±	−	−	±	+	−	±	+	−	±
34	±	−	±	+	+	±	±	−	−	+
35	±	±	−	+	+	±	−	+	±	+
36	−	±	−	+	+	±	±	±	−	+
37	±	−	−	+	+	±	±	±	±	+

TABLE II—*Concluded*.

Susceptible Mouse No.	Spleen.						Lymph nodes.			
	Size of nodules.	Lymphoid tissue in pulp.	Mitosis in lymphoid tissue.	Necrosis in lymphoid tissue.	Blood.	Pigment.	Mitosis in lymphoid tissue.	Necrosis in lymphoid tissue.	Lymphocytes in pulp spaces.	Endothelial cells in pulp spaces.
38	+	−	+	±	+	±	±	+	−	+
39	−	−	−	+	+	±	±	−	±	±
40	±	−	−	±	+	±	−	+	−	±
41	+	±	±	±	−	±	−	±	+	±
42	+	±	±	+	+	±	±	±	−	+
43	±	±	±	+	±	−	−	+	−	+
44	±	±	±	+	+	±	−	±	−	+
45	−	±	−	±	+	±	±	+	±	+
46	±	±	−	+	±	±	−	+	±	+
47	±	±	±	+	−	±	−	±	+	±
48	±	−	−	+	±	±	−	±	±	+
49	−	−	−	+	+	±	−	±	+	+
50	−	−	−	±	+	+	−	−	±	±
51	±	−	−	+	+	±	±	±	+	+
52	±	±	−	+	±	±	−	±	+	±
53	±	−	±	±	±	±	−	±	−	±
54	±	−	−	+	+	+	−	±	±	+
55	±	−	−	+	+	±	−	−	−	±
56	±	±	−	+	+	±	−	±	±	±
57	−	+	−	±	−	−	−	±	±	±
58	±	−	±	+	+	±	±	±	−	±
59	±	−	−	+	+	±	−	±	−	+
60	−	−	−	+	+	+	±	±	±	+
61	±	−	−	+	±	±	±	±	±	+
62	−	±	−	+	±	±	±	±	−	+
63	±	−	±	−	±	±	±	−	−	+
64	−	±	−	+	±	+	±	±	−	+
65	+	−	+	±	±	±	±	±	±	+
66	±	−	−	+	±	±	−	+	−	±
67	±	−	±	±	±	±	±	+	+	±
68	±	±	±	±	−	±	−	±	±	+
69	−	−	−	±	±	±	±	±	−	+
70	±	−	−	±	−	−	±	−	±	+
71	±	−	−	±	−	±	−	±	±	±
72	±	−	±	±	±	±	−	±	−	±
73	−	−	−	+	+	±	−	+	−	±
74	−	−	−	±	+	±	−	±	−	+
75	±	−	−	±	+	+	−	+	−	±
76	−	±	±	±	−	+	±	−	±	±

TABLE III.

Percentage of Immune Mice and Susceptible Mice.

Organ.	Points of difference.		Immune mice.	Susceptible mice.
			per cent	*per cent*
Spleen.	Size of nodules.	+	69	7
		±	24	47
		−	7	46
	Lymphoid tissue in pulp.	+	41	5
		±	55	39
		−	4	56
	Mitosis in lymphoid tissue.	+	28	4
		±	55	30
		−	17	66
	Necrosis in lymphoid tissue.	+	10	39
		±	55	50
		−	35	11
	Blood.	+	7	42
		±	58	38
		−	35	20
	Pigment.	+	4	13
		±	55	69
		−	41	18
Lymph nodes.	Mitosis in lymphoid tissue.	+	21	0
		±	58	36
		−	21	64
	Necrosis in lymphoid tissue.	+	0	26
		±	42	51
		−	58	23
	Lymphocytes in pulp spaces.	+	45	11
		±	52	50
		−	3	39
	Endothelial cells in pulp spaces.	+	10	47
		±	62	49
		−	28	4

In Table III a summary is given of the percentage of the animals in the susceptible and resistant groups showing the various histological changes indicated above.

DISCUSSION.

The point to be emphasized in the present study is the difference in the nature of the changes in the lymphoid organs of immune and of susceptible mice. In immune mice there are more or less marked indications of lymphoid hyperplasia, whereas a lymphoid depletion in varying degree tends to arise in susceptible mice. The former condition resembles greatly the histological picture which has been described in animals in which artificial lymphoid stimulation has been rendered.[2-5] The extreme cases of the latter or susceptible type conform in general nature with the condition induced by a large dose of x-rays,[8] although the cellular destruction is far less extensive. The effect is more like that seen after a long exposure to x-rays of low penetration.[4] These findings are in entire agreement with the results of previous experiments in which the importance of the part played by lymphocytes in artificial resistance to transplanted cancer in mice was pointed out.[5] They harmonize also with the observations of Mottram and Russ[9] who have shown that the spleens of rats resistant to the Jensen rat sarcoma tend to show higher lymphocytic content than the spleens of normal animals.

SUMMARY.

The lymphoid organs of mice show definite changes after cancer inoculation. In immune mice there is a tendency towards a lymphoid hyperplasia, while in susceptible mice more or less marked depletion of the lymphoid tissue takes place. These changes are evident at the end of the 3rd week after cancer inoculation.

[8] Heineke, H., *Mitt. Grenzgeb. Med. u. Chir.*, 1904–05, xiv, 21.

[9] Mottram, J. C., and Russ, S., *Proc. Roy. Soc. London, Series B*, 1917–18, xc, 1.

EXPLANATION OF PLATES.

PLATE 24.

FIG. 1. General histological appearance of the spleen of a mouse naturally immune to transplanted tumor.   A low power view.

FIG. 2. Germ center of the spleen of an immune mouse, showing numerous mitotic figures (*M*).

PLATE 25.

FIG. 3. General histological appearance of the spleen of a mouse susceptible to transplanted tumor.   A low power view.

FIG. 4. Deposits of pigment in the spleen of a susceptible mouse.

PLATE 26.

FIG. 5. Lymph node of a naturally immune mouse, showing abundant lymphocytes in the pulp spaces and a few mitotic figures in the lymph cords (*M*).

FIG. 6. Lymph node of a susceptible mouse, showing the proliferation of endothelial cells in the pulp spaces and pycnotic cells in the lymph cords.
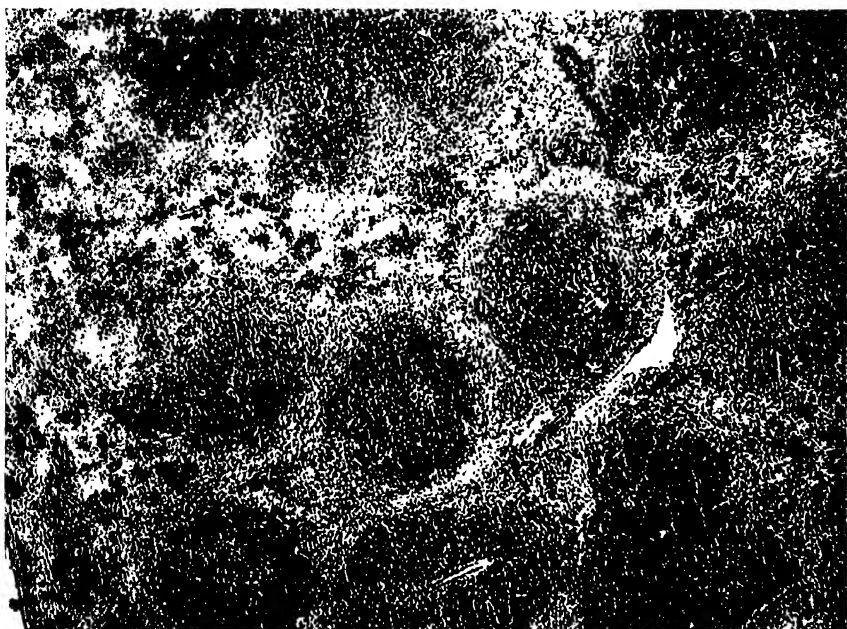
Fig. 1.

Fig. 2.
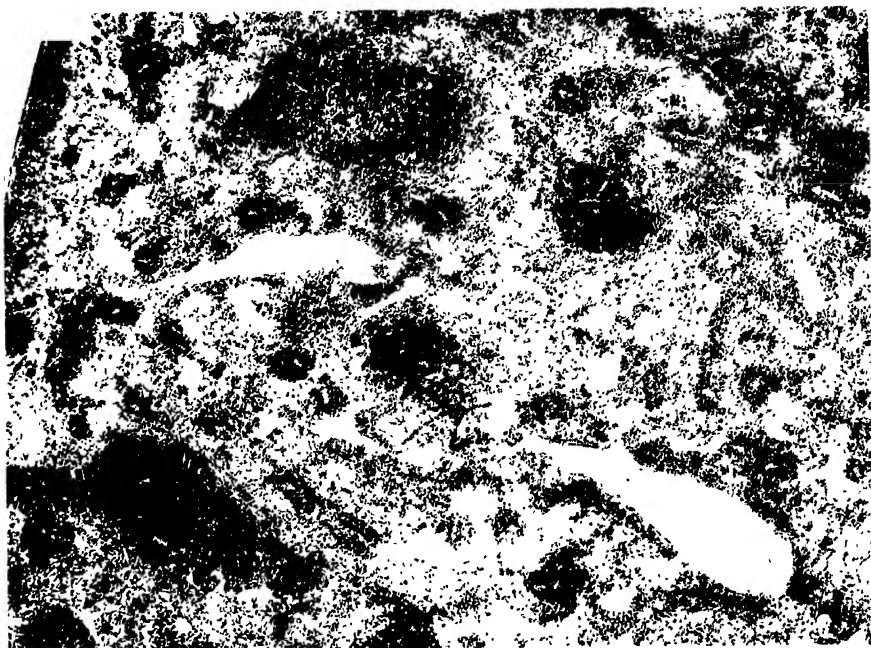
FIG. 3
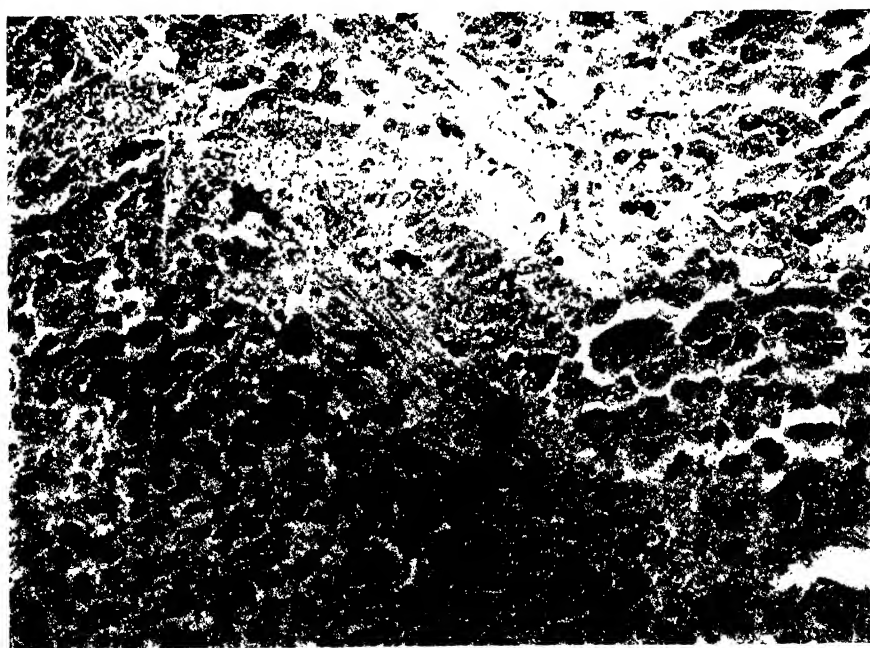
FIG. 4.

Fig. 5.

Fig. 6.

(Nakahara and Murphy: Resistance to transplanted cancer. VI.)

GENERAL LEUCOCYTIC RESPONSE OF THE GUINEA PIG DURING THE REACTION OF ARTIFICIAL IMMUNITY IN EXPERIMENTAL TUBERCULOUS INFECTION.

BY RAYMOND G. HUSSEY, M.D.

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Since the early studies on immunity reactions it has been known that a definite response of the circulating leucocytes is associated with the process of active immunity. The studies of Zinsser and Tsen,[1] which elaborate Bordet's original observation, furnish experimental evidence of the fact. In clinical medicine, also, it is known that a general leucocytic response accompanies the reaction of the body to bacterial infection. This response is characterized by quantitative changes in the actual number of circulating leucocytes, or in the relative proportion of the various types of cells, or in both.

Despite the fact that these observations are of long standing and are generally accepted, little is known of the actual significance of such changes. It may be stated, however, that for many infections the nature of this phenomenon is taken to indicate the degree of resistance possessed by a given individual. On the other hand, there are some bacterial infections about which there is controversy as to whether there is a characteristic leucocytic response. Hence, there is doubt whether observations of this phase of the blood picture are of value in the experimental or clinical study of such infections. Among these, active tuberculous infections are prominent. The studies of Murphy and his coworkers[2] in this laboratory, and those of Webb, Williams, and Basinger,[3] present evidence to show that the

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[1] Zinsser, H., and Tsen, E., *J. Immunol.*, 1917, ii, 247.

[2] Murphy, Jas. B., and Ellis, A. W. M., *J. Exp. Med.*, 1914, xx, 397. Taylor, H. D., and Murphy, Jas. B., *J. Exp. Med.*, 1917, xxv, 609. Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 35.

[3] Webb, G. B., Williams, W. W., and Basinger, A. F., *Tr. Nat. Tuberc. Assn.*, 1910, vi, 279.

resistance of animals to experimental tuberculous infection is enhanced when artificial lymphocytosis is induced before and after the inoculation of virulent tubercle bacilli. These studies support the observation of several clinicians[4] who have noted in human tuberculous disease that the circulating lymphocytes vary directly with the degree of resistance to the disease manifested by a given individual.

In previous communications from this laboratory, evidence is presented to show that lymphocytosis may be dependent upon the activity of germ centers in lymphoid tissue,[5] and in this sense lymphoid activity may be used as a corollary to lymphocytosis. On account of the interest and importance of these experimental and clinical observations and because the idea suggested by them is not generally accepted, it has seemed worth while to submit the subject to further experiment. We have accordingly made observations on the circulating leucocytes of guinea pigs in which resistance to virulent tubercle bacilli was raised by a previous inoculation of relatively non-virulent tubercle bacilli. We assume that animals so treated are possessed of as great a potential resistance to progressive tuberculous infection as is possible to obtain at present.

Method.

The method of protecting the animals against virulent tuberculous infection is that first carried out by Trudeau[6] and subsequently elaborated by Baldwin, Krause, and others. A preliminary inoculation of non-virulent tubercle bacilli, Saranac Strain R1, is followed, after a proper interval of time, by an inoculation of virulent tubercle bacilli, Saranac Strain H37.[7] As controls some animals were inoculated with Strain R1 or H37 alone. Guinea pigs were used throughout the observations.

[4] Ullom, J. T., and Craig, F. S., Am. J. Med. Sc., 1905, cxxx, 389. Webb, G. B., and Williams, W. W., Tr. Nat. Tuberc. Assn., 1909, v, 231. Wright, B. L., and King, R. W., Am. J. Med. Sc., 1911, cxli, 852. Solis-Cohen, M., and Strickler, A., Am. J. Med. Sc., 1911, cxlii, 691. Gutstein, M., Z. Tuberk., 1916, xxvi, 336.

[5] Nakahara, W., and Murphy, Jas. B., Anat. Rec., 1921 (in press).

[6] Trudeau, E. L., Tr. Assn. Am. Phys., 1903, xviii, 97.

[7] Acknowledgment is made of our appreciation to the workers at the laboratories of the Trudeau Foundation and Sanatorium for furnishing the cultures of Strains R1 and H37.

Absolute and differential leucocyte counts were made twice weekly on a number of normal guinea pigs over a period of a month. These blood specimens, as well as all others to be referred to, were obtained from a vessel of the ear. For the absolute counts the blood was diluted as usual with 3 per cent acetic acid in a diluting pipette and then shaken. 1 drop of the diluted blood was placed on the disk of a Türck hemocytometer, covered, and allowed to stand long enough for the cells to settle. Eight squares were always counted, and when the deviation of any given square was ten greater or less than the least number of cells obtained for any square, another drop was counted. The diluting pipettes were roughly calibrated before beginning the observations by making comparative counts. The same pipette was used for a given animal for each successive observation. For the differential counts blood films were prepared on cover-slips, then stained with Wright's stain. 300 cells were counted and the percentage of each type determined from this number was applied to the absolute count to estimate the absolute differential count.

During the period of preliminary observation any animal showing an erratic tendency in the leucocyte count was discarded. The general living conditions were the same for all animals. Therefore, when the experiment was begun, conditions were standardized as far as controllable variables permit, and were maintained throughout the observations.

A number of animals were inoculated subcutaneously into the left groin with 0.2 cc. of a suspension of Strain R1 tubercle bacilli prepared so that a stained film preparation showed not more than ten individual bacilli to a microscopic field. Blood counts were continued in the manner previously described. After about 3 weeks these animals were inoculated with 0.1 cc. of a suspension of the virulent strain, No. H37, and blood counts were continued. This suspension was standardized in the same way as the R1 suspension, except that the number of bacilli was not more than two to a microscopic field.

OBSERVATIONS.

For normal guinea pigs weighing between 200 and 250 gm. the average absolute count was 5,247 leucocytes per cc. of blood. Differential counts showed 52.1 per cent, or 2,734 of these cells, to be lymphocytes and 43.1 per cent, or 2,261, to be amphophils (polymorphonuclear cells).

Following the inoculation of Strain R1 the reaction was characterized by a gradual but definite increase in the total leucocyte

No. of weeks after R1 1 2 3
  " " " " H37   1 2 3 4 5 6
TEXT-FIG. 1.
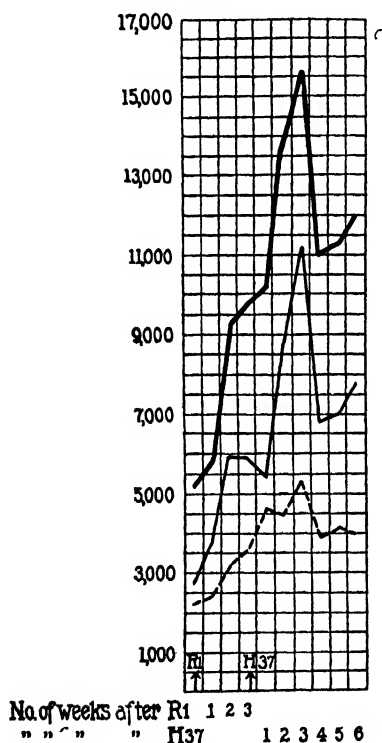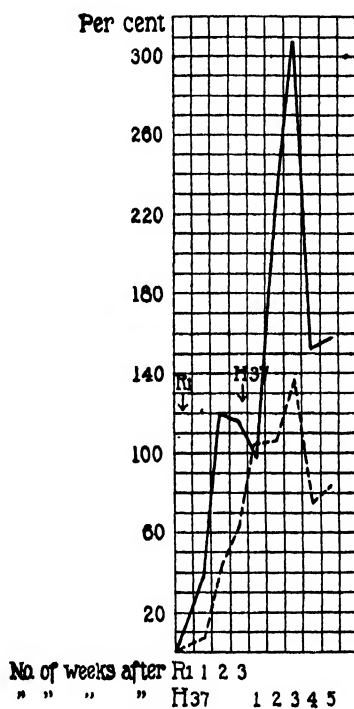
No. of weeks after R1 1 2 3
  " " " " H37   1 2 3 4 5
TEXT-FIG. 2.

——————— Absolute count.
————— Lymphocytes.
----------------- Polymorphonuclears.

TEXT-FIG. 1. Composite absolute and differential white cell counts of all test animals plotted at intervals of 1 week following inoculation and reinoculation.

TEXT-FIG. 2. The percentage variation of white cells following inoculation and reinoculation. The standard average of 2,734 lymphocytes and 2,261 polymorphonuclears is indicated as zero.

counts (Text-figs. 1 and 2).   At the end of 3 weeks, just before the inoculation of Strain H37, this increase was 80.5 per cent, representing an increase in lymphocytes to 60.5 per cent, while the polymorphonuclear cells, though increased absolutely, constituted 34.6 per cent of the white cells, a relative decrease of 8.5 per cent.

The reaction of the protected guinea pigs to the virulent strain, No. H37, was characterized first by a slight fall in the lymphocytes and an
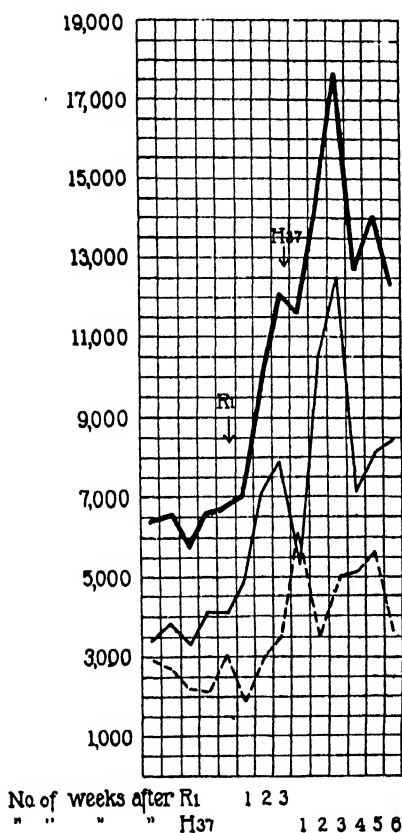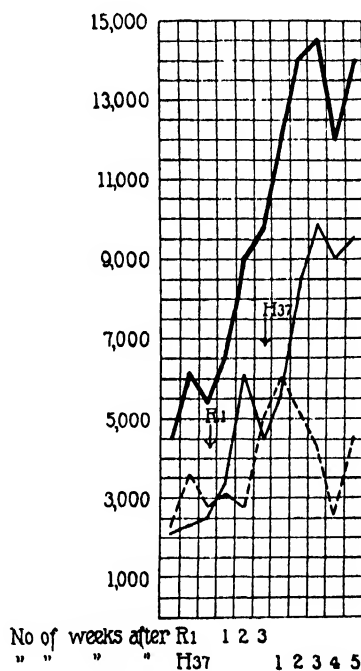
TEXT-FIG. 3.   TEXT-FIG. 4.

————————— Absolute count.
————————— Lymphocytes.
----------------- Polymorphonuclears.

TEXT-FIGS. 3 and 4. Absolute and differential white cell counts of two individuals used in the experiment, plotted at weekly intervals before and after inoculation and reinoculation.

increase in the polymorphonuclear cells.   This was observed throughout the 1st week, during which time the animals were obviously quite sick.   Then there followed an exaggerated increase in the total leucocyte count, which reached a peak during the 3rd week, at which time the total percentage increase of white cells amounted to 198.5. During this period the lymphocytes increased to a point 306 per cent above their number at the beginning of the experiment, while the polymorphonuclear cells were 9.2 per cent below their normal relative

No of weeks after H37 1 2 3 4 5

TEXT-FIG. 5.

No of weeks after H37 1 2 3 4 5
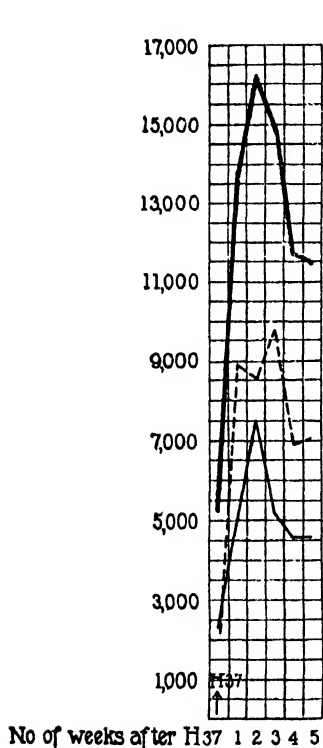
TEXT-FIG. 6.

——————— Absolute count.
——————— Lymphocytes.
----------------- Polymorphonuclears.
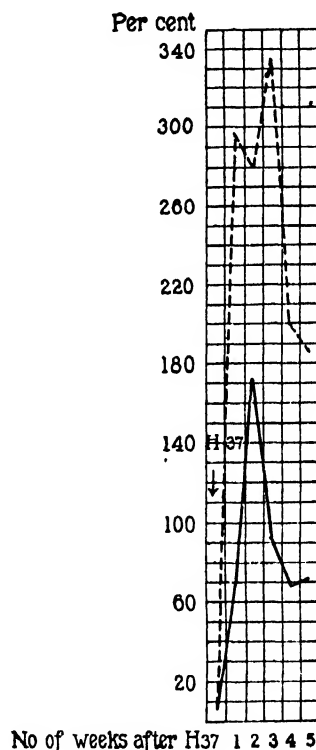
TEXT-FIG. 5. Composite absolute and differential white cell counts of individuals plotted at weekly intervals following an inoculation of a heavy suspension of virulent tubercle bacilli.

TEXT-FIG. 6. The percentage variation of white cells in the counts plotted in Text-fig. 5.

average, though numerically they showed some increase. After the peak of the curve was reached, there was a decided drop in the absolute number of white cells, particularly affecting the lymphoid cells. Text-figs. 3 and 4 show typical curves of individual animals used in the experiment. In Text-fig. 4 it will be noted that the normal count shows the polymorphonuclear cells to be greater in number than the lymphocytes, an exception occasionally observed.

Blood counts on the control guinea pigs inoculated with virulent tubercle bacilli alone showed a definite increase in the circulating leucocytes. The relation between the increase of polymorphonuclear and lymphoid elements was erratic. Immediately following inoculation both types of cells increased in about the same numerical proportion. During the 2nd and 3rd weeks the lymphocytes showed a greater increase; after this time there was a progressive decrease in lymphocytes with a corresponding increase in polymorphonuclear cells.

Ten guinea pigs were inoculated with a much heavier suspension of Strain H37. The counts on these animals are shown in Text-figs. 5 and 6. They demonstrate a distinct difference in the reaction to more massive inoculation of virulent tubercle bacilli in contrast to the response to the smaller dose.

DISCUSSION.

In the observations recorded it is to be noted that evidence of lymphoid activity varies directly with the resistance to progressive tuberculous infection shown by the animals used. Such facts support the views previously quoted regarding this parallelism. It seems apparent, however, that one is not justified in attempting an explanation of this parallelism since little information is available concerning the function of lymphocytes.

The general idea of some such relation is not new, since as early as 1883 Arloing[8] developed the notion during an attempt to bring experimental evidence to bear on the controversy of that time between clinicians and pathologists concerning the tuberculous nature of scrofula. This investigator and others who followed his lead believed that lymphoid cells possessed some specific property which was

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[8] Arloing, S., Leçons sur la tuberculose, Paris, 1892.

active in the defensive mechanism against tubercle bacilli. These opinions were based largely upon observations made on the differences in the reaction of rabbits following the inoculation of material from tuberculous lymphadenitis as compared to that from other tuberculous tissues. Most of this work was done before it was known that there are bovine and human strains of tubercle bacilli, which probably accounted for the observations recorded. Renewed interest came later through the work of Bartel and coworkers who claimed that, while working on experimental tuberculous infection in guinea pigs induced by feeding tubercle bacilli, they observed in lymph nodes in which tubercle bacilli had lodged, without further evidence of invasion, a reaction characterized by a striking lymphoid hyperplasia.[9] Because of the absence of both the characteristic mononuclear phagocyte and the giant cell, they concluded that lymphocytes alone were capable of destroying tubercle bacilli. Further experiments failed to confirm their views. It has been thought that a specific substance might be isolated from lymphoid tissue and used for artificial immunization, but the failure of experiment to support any of these ideas has led to hesitancy in the acceptance of any lead in the study of resistance to tuberculous infection which concerns lymphoid activity.[10] However, we regard the fundamental idea in the studies initiated in this laboratory concerning lymphoid activity in tuberculous infection[2] as totally different from any of those just mentioned. The facts brought out in these observations seem significant, and we believe that they emphasize a probable relation between lymphoid activity and resistance to experimental tuberculous infection which deserves consideration in the study of the factors of resistance to active tuberculous infection in man.

It may be said that several investigators have recorded data which show that the nature of the leucocytic reaction of individuals ill with tuberculous infection in its various forms is analogous to that recorded in this paper. For example, in generalized miliary tuberculosis and in tuberculous meningitis, in which resistance is lowest, since recovery is always improbable, there is a notable deficiency in lymphoid activity as determined by estimations of circulating lymphocytes. In progressive pulmonary tuberculosis this is also the case; on the other hand, in individuals showing an ability to control the disease, there is an increase in the circulating lymphocytes. Although there is no general agreement among various workers that these statements are correct, the disagreement is no more extensive than that concerning the leucocytic reaction in many other infections.

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[9] Bartel, J., and Neumann, W., *Centr. Bakt., 1te Abt., Orig.*, 1905–06, xl, 518.

[10] Krause, A. K., *Am. Rev. Tuberc.*, 1917–18, i, 717.

In this connection it seems of interest to direct attention to certain probable reasons for the differences in opinion; namely, confusion arising from observations made during different phases of a given illness associated with a particular infection, *e.g.* the period of typical manifestations of the infection, that of complications, and that of convalescence; conclusions drawn from data which include too few observations on different individuals; or lack of uniformity in the method of interpretation. These points are illustrated in the statement found in most text-books concerning the leucocytic reaction in general miliary tuberculosis, typhoid fever, and influenza. It is usually asserted that the leucocytic picture in the three infections is similar. However, careful analysis of reports on studies of these infections leads to quite a different conclusion. A summary of the tables in Warthin's paper[11] on general miliary tuberculosis and Thayer's paper[12] on studies on typhoid fever is as follows:

General Miliary Tuberculosis.

Absolute count.................. 4,128	Decrease........ 2,872 (41.03 per cent)
Polymorphonuclears............. 3,777	Relative increase....... 20.5 " "
(91.5 per cent)	Absolute decrease. 1,123 (22.39 " ")
Mononuclears................... 342	Relative " ...... 16.7 " "
(8.3 per cent)	Absolute " ..1,408 (30.45 " ")

Typhoid Fever.

Absolute count.................. 5,386	Decrease .... 1,614 (23.05 per cent)
Polymorphonuclears............. 3,323	Relative decrease....... 8.3 " "
(61.7 per cent)	Absolute " ..1,577 (32.18 " ")
Mononuclears................... 2,028	Relative increase....... 12.06 " "
(37.6 per cent)	Absolute " ....278 (15.89 " ")

The percentage estimations noted are made on the basis of a standard of 7,000 leucocytes per c. mm. of blood, with the percentage relation of polymorphonuclear cells and lymphocytes 70 and 25 respectively.

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[11] Warthin, A. S., *Med. News*, 1896, lxviii, 89.
[12] Thayer, W. S., *Johns Hopkins Hosp. Rep.*, 1900, viii, 489.

Further, the recent widespread epidemic of influenza has furnished an opportunity to gain much new information concerning the leucocyte picture found in that infection. Practically all observers affirm a leucopenia as characteristic, but there is difference of opinion regarding the relative proportion of the various cells. Some observers have noted the mononuclear elements to be especially affected,[13] while others pay particular attention to polymorphonuclear elements.[14] The reports which point to the mononuclear elements are based on observations made during the first 5 days of illness, while those which point to the polymorphonuclear cells are concerned with a later period of the illness. In view of the now generally recognized fact that true influenza is of only short duration (1 to 5 days) and that one deals principally with complications in an illness which continues beyond that period, it is suggested that the observations made during the early period should be of greater significance. The report of Olitsky and Gates[15] on experimental influenza lends support to this view. While it is true that all three infections manifest a leucopenia, there are striking differences demonstrated in the degree and character of leucocyte changes.

As further emphasis to the suggestion that more consideration should be given to the possible value of lymphocytes in the defensive mechanism against tubercle bacilli, attention is called to the constant presence of these cells in the local reaction product resulting from tissue injury by tubercle bacilli. Among the numerous investigators who have studied the histogenesis of the tubercle, there has been a tendency by the followers of Baumgarten to disregard the lymphocytes entirely, while those following Metschnikoff have regarded them as progenitors of the characteristic mononuclear phagocytes found in the lesions. It must be stated, however, that all these workers have concerned themselves principally with the controversy as to whether fixed or wandering cells enter chiefly into the reaction.

More recently, however, Wallgren,[16] in a study on experimental tuberculosis of the liver in rabbits, emphasized the part played by

[13] Alder, A., *Folia hæmatol.*, 1919–20, xxv, 16.

[14] Berger, W., *Beitr. Klin. Infectionskrankh.*, 1919–20, viii, 303.

[15] Olitsky, P., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.

[16] Wallgren, A., *Arb. path. Inst. Helsingfors*, 1911, iii, 139.

lymphocytes in the reaction of the tissues of this organ to tubercle bacilli, while von Fieandt,[17] in a similar study on the meninges, found little evidence of participation by lymphocytes in the process. These observations are of interest in connection with the established fact that different tissues of a host vary in their resistance to parasitic invasion. Soper[18] has demonstrated in the liver of the rabbit a relatively high degree of natural resistance to tubercle bacilli, and it is well known that the meninges are very susceptible to these parasites. Whether or not the success of such a local resistance may be related to a combined activity of the two types of mononuclear cells found in the product of reaction can only be conjectured at this time. Further elaboration of this point must be delayed until methods of experiment are available which will permit its investigation. In the meantime it would seem more plausible to assume that the presence of lymphocytes in these reactions is a purposeful phenomenon, as is believed for other types of cells in various reaction products following tissue injuries. Particularly is this true in the light of available information regarding the factors concerned in the mechanism of immunity in tuberculous infection. There is almost unity of opinion that as far as cellular and humoral elements are concerned, the former are conspicuous while the latter are relatively inconspicuous.

SUMMARY.

Guinea pigs have been rendered relatively immune against infection with virulent tubercle bacilli by preliminary inoculation with a suitable quantity of avirulent tubercle bacilli. Blood counts on these animals show that associated with the immune reaction there is a definite general leucocytic response characterized by an absolute increase in the total count, with an absolute and relative increase in the lymphocytes. The period of greatest activity coincides with that known to be the period of greatest reaction, based on anatomical evidence during the course of infection following this method of immunization. Moreover, blood counts made on animals inoculated with avirulent tubercle bacilli alone show an increase in the circulating

[17] von Fieandt, H., Arb. path. Inst. Helsingfors, 1911, iii, 235.

[18] Soper, W. B., Am. Rev. Tuberc., 1917–18, i, 385.

lymphocytes during the period of greatest reaction to the infection, while blood counts on guinea pigs inoculated with virulent bacilli alone show an erratic course in which the polymorphonuclear forms are much increased, though not regularly so.

These results indicate a parallelism between lymphoid activity and resistance of the animals to tuberculous infection, and suggest an association of lymphocytes with the factors determining this resistance, a relation which warrants consideration of the blood picture in the clinical study of tuberculous infection.

TEST FOR CHANGES IN THE PROTEIN CONTENT OF THE CEREBROSPINAL FLUID.

Based on the Flocculation of Lipoids.

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The several roughly quantitative, clinical methods now in use for the detection of pathologically increased proteins in the cerebrospinal fluid may be divided into two groups, those in which heating is required, and those which are carried out in the cold. Of the former, the butyric acid reaction[1] has been considerably used because of its delicacy and the readiness with which the results can be read; but the unpleasant odor of the reagent has been a drawback to its use. Amoss[2] has proposed a method in which the native proteins of cerebrospinal fluid are precipitated by boiling for six minutes in a water bath a mixture of the spinal fluid (0.2 c.c.) and an aqueous solution (0.6 c.c.) containing primary phosphate (3 per cent.) and glacial acetic acid (0.05 per cent.). The latter method, while perhaps a trifle less sensitive than the butyric acid reaction, yields practically comparable results, and the use of a single odorless reagent is a distinct advantage. On the other hand, a method in which a single, odorless reagent is used, without heating, would constitute a still further gain in practice.

In the several nonheating procedures the contrast between normal and pathologic is less clear-cut than in the butyric acid reaction or Amoss' test. Such are the Nonne-Apelt[3] and the Ross-Jones[4] methods with saturated ammonium sulphate solution, and Pandy's[5] reaction

1. Noguchi, Hideyo: Serum Diagnosis of Syphilis, Ed. 3, Philadelphia, J. B. Lippincott Company, 1912.

2. Amoss, H. L.: A Test for Globulin in Spinal Fluid for Use at the Bedside, J. A. M. A. **72:** 1289 (May 3) 1919.

3. Nonne and Apelt: Arch. f. Psychiat. **63:** 433, 1907.

4. Ross and Jones: Brit. M. J. **1:** 1111, 1909.

5. Pandy: Neurol. Zentralbl. **29:** 915, 1910.

with concentrated (1:16) phenol (carbolic acid). Trichloracetic acid and sulphosalicylic acid have also been used as precipitating reagents, but as they bring about precipitation in normal as well as pathologic fluids, careful quantitative measurement of the precipitated proteins is necessary. Such quantitative tests with sulphosalicylic acid have been recommended by Ravaut and Boyer[6] and by Denis and Ayer.[7]

It is evident, then, that none of the methods so far used is without drawbacks; hence the attempt to devise a method combining several advantages—the use of a single reagent which can be simply applied (without heating) and is capable of preservation; delicacy, rapidity, and distinctness of reaction.

As will be evident from the constituents of the reagent, the reaction is not altogether based on the direct precipitation of the proteins contained in the cerebrospinal fluid, but is due to a concomitant flocculation of certain lipoids which the reagent contains. The volume of the precipitate produced is therefore more copious than that brought about by the direct precipitation methods, such as the butyric acid, Amoss', Pandy's or Esbach's methods.

The Reagent and its Mode of Application,

The components of the reagent are (1) alcoholic extract of acetone-insoluble tissue lipoids prepared by the method to be described, and (2) a solution containing 0.5 c.c. of glacial acetic acid, 10 c.c. of a saturated solution of picric acid in absolute alcohol, 1.5 gm. of acid potassium phosphate, and 4 gm. of sodium chlorid in distilled water to make the total volume 1,000 c.c. The lipoidal extract and the acid-salts solution, which will be referred to, respectively, as Solution 1 and Solution 2, are later mixed in the manner to be described.

Preparation of the Stock Solution of the Acetone-Insoluble Lipoids (Solution 1).— Beef heart is ground in a sausage machine, and then completely dried by a fan over a heater. One hundred grams of the dried substance are extracted with 1 liter of acetone for five days at room temperature, with daily shakings. The

6. Ravaut, P., and Boyer, L.: Presse med. **28:** 42 (Jan. 17) 1920.

7. Denis, W., and Ayer, J. B.: Method for Quantitative Determination of Protein in Cerebrospinal Fluid, Arch. Int. Med. **26:** 436 (Oct.) 1920,

acetone is then discarded, and the mass of solids freed from acetone by evaporation and then extracted with 1 liter of absolute alcohol for five days at room temperature. The golden yellow alcoholic extract, which contains acetone-insoluble tissue lipoids (which has been found also to be an excellent antigen in the complement fixation test for syphilis), is separated from the dried muscle and tested for its suitability as the reagent. The criterion of usefulness is the transparency of a mixture of the alcoholic extract and Solution 2 in a ratio of 1:9. If a marked opalescence bordering on opacity is produced, the extract is unsuitable. As a rule a satisfactory solution—faintly opalescent, but almost transparent—is obtained.

Preparation of Solution 2.—It is convenient to prepare a stock solution of the acids and salts in distilled water as follows: One and five-tenths grams of acid potassium phosphate (KH_2PO_4) and 4 gm. of sodium chloride are dissolved in 990 c.c. of distilled water containing 0.5 c.c. of glacial acetic acid. Finally, 10 c.c. of a saturated solution (approximately 3.5 per cent.) of picric acid in absolute alcohol is added. If the solution is not to be kept in the refrigerator it is desirable, in order to prevent any fungus growth, to make it up in ten times the strength in which it is to be used, that is, to dissolve the salts in 90 c.c. of distilled water, containing 0.5 c.c. of glacial acetic acid, and 10 c.c. of the picric acid solution. At the time of use, 1 part of this solution should be mixed with 8 parts of distilled water, and 1 part of the lipoidal solution added.

Preparation and Preservation of the Reagent.—To 9 parts of Solution 2 is added gradually, mixing by gentle agitation, 1 part of the alcoholic lipoidal extract (Solution 1), the resulting mixture being faintly opalescent, almost transparent. (If the process is reversed and Solution 2 poured into Solution 1, a turbidity results which makes the reagent unsuitable.) The mixture in this form has been found to remain unchanged for a period of several weeks and will perhaps prove not to be subject to deterioration; but as the two solutions may be preserved separately for an indefinite period, the reagent can be made fresh from time to time as needed.

It must be mentioned that the ordinary alcoholic extract of beef heart with or without the addition of cholesterin (so-called syphilitic antigens used by some serologists) does not give a useful reagent, but one too sensitive, readily producing precipitation even when mixed with normal spinal fluid. An alcoholic extract of the dried beef heart muscle without preliminary acetone extraction is likewise unadapted, owing to the presence of certain acteone-soluble lipoids (cholesterin, etc.). The addition of cholesterin to a suitable alcoholic extract of acetone-insoluble tissue lipoids, as already described, renders the latter too sensitive and therefore worthless as the reagent.

A differentiation can be made between pathologic and normal spinal fluids without the addition of the salts, acid potassium phosphate and sodium chlorid, to Solution 2; but their presence in the concentrations indicated makes the readings easier and more certain by causing greater opacity of the flocculated lipoids.

Mode of Application.

Into a small test tube such as ordinarily used for the writer's system of the Wassermann reaction (10 by 1 cm.) is measured 0.1 c.c. of cerebrospinal fluid, and 1 c.c. of the reagent is then added. A normal spinal fluid remains perfectly clear or becomes only faintly opalescent, while a dense general turbidity is produced in all specimens containing an increased amount of globulin or albumin. In cerebrospinal fluids from bacterial meningitis the flocculation is dense and copious, followed by complete or partial precipitation of the granular flocculi within about an hour at room temperature. Specimens from general paresis and tabes dorsalis give a dense flocculation, somewhat less copious than those from acute inflammatory conditions, the granular flocculi settling to the bottom of the tube within a few hours.

The reaction is rapid, the maximum opacity being reached within a few minutes; the granulation and subsequent sedimentation of the flocculi, however, require a longer time. The reaction takes place at any temperature from that of the icebox to that of the incubator, but room temperature has been found most satisfactory. If haste is necessary, the reaction can be read within ten minutes; but a supplementary reading after the tubes have been left at room temperature for several hours or over night is advisable. A mild reaction is indicated by a slight general turbidity, best recognized in reflected light.

The method has been applied to a large number of specimens of cerebrospinal fluid, and comparative observation shows that the test runs parallel with the butyric acid reaction and hence promises to be a useful addition to the diagnostic methods applied to the cerebrospinal fluid.

Quantitative determination of the protein contents of the spinal fluid can also be made with this reagent. The details of the procedure are reserved for later publication.

THE DUALISTIC CONCEPTION OF THE PROCESSES OF ANIMAL LIFE.

By S. J. MELTZER, M.D.

Animal life is manifested by an uninterrupted stream of various forms of perceptible activities, harmoniously performed by the various groups of the living tissues which enter into the make-up of the unit of a living organism. The multitude of the life phenomena may be reduced in a general way to three elements: motion, secretion and sensation. This classification may not be exhaustive but it is all sufficient for the object of this presentation. I wish, however, to emphasize that in most instances, if not in all, the perceptible activities are discontinuous; the activities alternate with periods of rest. The following examples will illustrate this point. Each cycle of a heart-beat is interrupted by a diastole when the heart muscle appears to be at rest. Or the peristaltic movements in one series of segments of the digestive tract, or of other tubes within the animal body, are accompanied by a more or less prolonged quietude of other segments of these tubes. Skeletal muscles contract at irregular intervals and only at the behest of some internal or external stimulus. Glands with external secretions functionate only at intervals and only as a result of certain provocations; for instance, the salivary glands, the digestive glands of the stomach and intestines prepare and empty their products in the corresponding cavities only when they are provoked by the entrance of food or by psychical stimuli. The same holds true for the sensory sphere; definite sensations are perceived only when certain receptive end-organs are affected by adequate stimuli.

Since the discovery of the circulation by Harvey, or perhaps, more correctly, since the discovery by Haller, that irritability is a distinguishing property of all living tissues, physiologists have been busily engaged in discovering the causes for and the mechanisms of the various activities of the living animal organism. Two sets of nerves were discerned; one set carries impulses in the centripetal

direction, from a receptive end-organ to the central nervous system or to prevertebral ganglia, and another set of nerves carries impulses from the central nervous system or from prevertebral ganglia to peripheral effective end-organs. The impulses transmitted by the centripetal nerves either cause well-defined sensations in various parts of the brain or cause organized nerve centers to send out reflexly impulses in centrifugal directions. The centrifugal nerves carry impulses either to motor mechanisms or to secreting glands.

Recently it was discovered that the circulation is carrying chemical stimulating substances from one part of the body to another without the intermediation of the nervous system. These substances which are manufactured by specific organs or tissues are now known as hormones.

Physiology in the past devoted its energies to the study of the causes and the mechanisms of the active part of living phenomena, and little or no attention was paid to the resting phase of these phenomena. On the surface of things this attitude would seem justified and logical. Only an action calls for an explanation, requires the discovery of a cause. Diminution of an action or its entire disappearance is readily explained by the reduction in the extent or intensity of the cause or by its complete elimination.

Are living tissues when they are inactive in a state of inanimateness? Seventy-five years ago a fact of first magnitude was discovered which permits, indeed compels one to look at life phenomena and at living tissues from a new angle. It is an undisputed fact that stimulation of the peripheral end of a motor nerve causes a contraction of the corresponding muscle which subsides with the discontinuation of the stimulation. The vagus nerve was considered as the motor nerve of the heart muscle. Now, in 1845, the brothers Weber discovered that stimulation of the peripheral end of a cut vagus causes the heart to stop in diastole. Let me add here that it was found later that during this standstill the heart muscle is more relaxed than during the normal diastole and that at the same time its normal irritability or conductivity are diminished. Here was a new phenomenon, rest of a muscle is being brought about by an active cause, by a stimulus. The Webers designate the cardiac vagus as an inhibitory nerve. In the following half-dozen years

numerous attempts were made to explain the cardiac standstill by all sorts of assumptions, but with no avail; inhibition came to stay. (Permit me to bring to your attention the following historical incident which is an interesting contribution to the psychology of investigators: A. W. Volkmann, a celebrated physiologist in his time and a brother-in-law of the Webers, investigated systematically the various nerves of the animal body as to their nature, whether they are sensory or motor nerves. While investigating the relations of the vagus nerve of frogs to the heart, he stimulated these nerves by turning a wheel of a faradic apparatus and counted the heartbeats. He found that before the stimulation the heart was beating twenty-six times per minute, while after the discontinuation of the stimulation the heart was beating thirty-one times per minute. From this he concluded that the vagus is a motor nerve of the heart muscle. He added: singularly enough the heart stopped beating during the time of stimulation. Four years later Volkmann discarded the entire value of his observation, under the assumption that muscles which contract spontaneously are not proper subjects for investigation. But three years later the Webers saw the same fact of the stoppage of the heart-beat during stimulation, and had the courage to pronounce it a new phenomenon in physiology.)

I may mention here that the inhibitory action of the vagus upon the heart was later shown to exist in human beings. Czermak succeeded in stopping his heart by compressing the vagi in the neck. On the other hand it was shown that the inhibitory nerve fibers of the vagi are moderately stimulated continuously in man and in certain animals by means of a center in the medulla which is in a state of tonus. The administration of atropin paralyzes the nerve endings of the inhibitory vagus and thus accelerates the heart-beats.

Is it logical to assume that the cardiac vagus demonstrates the only inhibitory phenomenon in the animal body? In the course of the three-quarters of a century which passed since the discovery of the vagus action upon the heart, numerous inhibitory phenomena have been discovered. The time at my disposal is by far too short to attempt to enumerate all the new facts which bear upon this question. But I shall try to mention a few. In the first place I may mention one fact which the speaker was instrumental in dis-

covering. · Forty years ago I studied the physiology of deglutition. It was established that after the contraction of the mylohyoid muscle and the elevation of the larynx which characterize the initial act of swallowing, a characteristic peristaltic movement runs down the esophagus and reaches the cardia in about six seconds. The question arose, What happens to this peristaltic wave in the esophagus when deglutitions were repeated every second or two? The answer was a surprise. After frequent swallows the peristaltic wave of the esophagus sets in only after the last swallow. This fact and other observations prove conclusively that in the complex reflex acts of deglutition, with each swallow an inhibitory impulse runs ahead of the impulse which causes the progress of the peristaltic wave in the esophagus. Animal experimentation demonstrated that both impulses run not in the periphery but in the center of deglutition. It was further discovered that during the act of deglutition the inhibitory impulse irradiates to neighboring centers within the medulla. It inhibits the tonus of the cardiac vagus, the vasomotor tonus and the activity of the respiratory center. These findings present a definite evidence that inhibition is an integral part of a normal function of the animal body.

'Permit me here to make a side remark. Most of the physiologic experimentations have to be made on animals while they are under anesthesia. But since anesthesia suppresses the activities of the center of deglutition I had to perform these experiments on myself. It was certainly not a great pleasure to have for many hours two stomach tubes, with distended balloons at their end, in my pharynx and in the esophagus. The experiments were carried on in the physiological institute of the University of Berlin. One day Herr von Goslar, then the Minister of Education in the Prussian Government, came to see these experiments. A few days later we learned the purpose of his errand. The Junker party introduced in the Prussian Diet a law to suppress animal experimentation. In reply to the old challenge that if physiologists are so anxious to contribute to science, why did they not make the experiments upon themselves, von Goslar told them what he saw in the physiologic laboratory, adding that he was sure that among the gentlemen of the extreme Right, with all their reputed courage, there was no one who would

be willing to perform such experiments upon himself for money or glory. That finished the vicious Junker attack upon the medical sciences. I tell this story because there are many hysteric people imbued with the same Junker spirit of enmity against the medical sciences.

The existence of inhibition was discovered in most of the manifestations of life. Most interesting are the findings in which the presence of the inhibitory factor facilitates the prompt functioning of a physiologic mechanism. In the past twenty-five years Sherrington studied many instances in which simultaneously with the contraction of one group of muscles the corresponding antagonistic group is inhibited. For instance, the contraction of the flexor group is accompanied by an inhibition of the extensor group of muscles, and *vice versa*, or the contraction of an adductor group is accompanied by a simultaneous inhibition of the corresponding abductor groups, and *vice versa*. Sherrington designated this mechanism as *reciprocal innervation*. In 1883 I called attention to the existence of the mechanism of reciprocal innervation in the respiratory function. During inspiration the inspiratory muscles contract while the expiratory muscles relax, and *vice versa*. Recent experiments on fowls by Dr. Arthur Meyer and myself brought out a striking illustration. Fowls have no diaphragm. The inspiration is caused by the contraction of the intercostal muscles. The expiration is accomplished by the contraction of the abdominal muscles. In these experiments the inspiratory muscles and the internal oblique muscle of the abdomen were prepared to record separately on a revolving smoked drum. It was very instructive to find that on stimulating the central end of one vagus the thorax was raised to an inspiratory phase, while the expiratory internal oblique muscle became relaxed. But the reciprocal innervation is only a part of a general law which I termed the Law of Antagonistic Innervation. As has been mentioned before, in the act of deglutition the lower part of the esophagus relaxes while the upper part contracts. Bayliss and Starling found that a stimulus applied to the intestine causes a contraction above and a relaxation below the point of stimulation. Joseph and I found that during a contraction of the pylorus a relaxation of the duodenum takes place. Cannon and Lyman observed that during the contraction of the

ileum the iliocecal valve relaxes.   The relaxation of all these segments of the digestive canal, which takes place simultaneously with the contractions of the segments above them, means that the lower part is inhibited, relaxed, in order that it may not offer an obstacle to the progress of the contents propelled forward by the contractions of the segments above.   But in these instances there is no reciprocity. The general law means that in each motor function an inhibitory factor is involved which removes any resistance that may be offered by antagonists.

The detection of inhibitory phenomena is surrounded with great difficulties because only in exceptional instances can they be reached by separate nerves.   But here again the extrinsic nervous mechanism of the heart offered a helping hand.   About fifty odd years ago Ludwig and Bakst discovered fibers in the sympathetic nervous system which are antagonistic to the inhibitory nerves of the vagus, that is, the stimulation of these nerves accelerates the heart beats and augments the systolic contraction.   During this effect the irritability and conductivity of the heart muscle is increased.   This bundle of nerves is known as accelerators.   There are certain characteristic differences between the inhibitory nerves of the vagus and the accelerators.   Thus the vagus has only a short latent period while the accelerators have a very long latent period—that is, the nerves have to be stimulated a long time before they develop their effect upon the heart.   Furthermore the vagus has only a short after-effect while the accelerators have a very long after-effect—that is, after discontinuation of the stimulation the accelerating effect may continue for a minute and longer.   Now if both kinds of nerve fibers are stimulated simultaneously, interesting phenomena make their appearance.   During the stimulation practically no other effect is recognized but the inhibition of the heart-beats, but quite soon after discontinuation of the stimulus the heart starts beating at a rate as if there had been no other stimulation but that of the accelerator nerves.   That means that although during the simultaneous stimulation the accelerator seemed to exert no influence, the effect upon that nerve was merely covered but not annihilated, and it exhibited this influence after discontinuation of the stimulation in a manner as if it had been stimulated all alone.   These facts helped

to demonstrate the presence of accelerator fibers in the vagus nerve of the frog. Besides the differences mentioned there are other conditions which distinguish the two kinds of nerve fibers. They differ, for instance, in fatigability and in their behavior toward the effects of temperature.

Now the overwhelming immediate effect of the inhibitory nerves and the long after-effect of the accelerator nerves is not peculiar to the inhibitory or the accelerating side of these nerves. This can be seen in the effect of the chorda tympani and sympathetic nerves to the bloodvessels of the salivary glands. The bloodvessels in general are under the influence of peripheral antagonistic nerves, nerves the stimulation of which causes a contraction of the bloodvessels, and others the stimulation of which causes a dilatation of the bloodvessels, vasoconstrictors, and vasodilators. The vasodilators are considered as vascular inhibitory nerves. Now the chorda tympani nerve, which causes a dilatation of the bloodvessels of the submaxillary salivary glands, possesses a long after-effect, and the sympathetic nerve which causes a constriction of these bloodvessels has only a very short after-effect. When both sets of nerves are stimulated simultaneously, during stimulation only a vasoconstriction can be noticed, but after cessation of the stimulus a long-lasting, strong, vasodilatation makes its appearance. In contrast to the conditions found in the extrinsic nerves of the heart, here the vasoconstrictor, which may be compared with the accelerator, exerts an overwhelming effect during stimulation, while the chorda, the inhibitory nerve, possesses the long after-effect which comes to the fore after discontinuation of the stimulus.

Dr. Githens and I recently found in the nose-licking reflex a clear example of the rôle which the overwhelming effect of one set of nerves and the after-effect of the antagonistic set of nerves may play in the mechanism of some reflexes. We first found that a certain procedure which leads to mechanical anesthesia abolishes completely all the sensations, leaving all the reflexes intact. Using this method of anesthesia, we discovered the nose-licking reflex. The tip end of the nose and the anterior part of the septum could be quite intensely compressed by means of a hemostatic forceps without producing any effect. But immediately on removal of the forceps

the animal started licking its nose, which lasted a few seconds. Evidently the pressure stimulated two sets of afferent nerves, one set which would cause the licking reflex and the other set which inhibits this reflex. During the pressure the inhibitory nerves prevailed, but after removal of the stimulus caused by pressure, the long after-effect of the antagonistic nerves was permitted to become manifest and the nose-licking made its appearance.

As seen above, the overwhelming effect of one set of nerves upon the antagonists, and the long after-effect of another set of nerves, are not exclusive properties of either inhibitory nerves or nerves of action. Nor do we need to expect that in all instances each of these properties or both are so extreme as they were found in the examples cited. We may, for instance, assume that in the contraction of the skeletal muscle of vertebrates there are inhibitory and motor impulses simultaneously at work. The motor impulses may be indeed overwhelming during the indirect stimulation of the motor nerve or direct stimulation of the muscle. But after cessation of the stimulus there is a moderately long after-effect of the inhibitory impulse so as to cause a rapid relaxation of the contraction of the muscle. The direct or indirect stimulation of a vertebrate striated muscle does not offer undisputed evidence of the presence of inhibitory impulses through the motor nerves or in the muscle substance itself. But veratrinized muscles of frogs show a very slow relaxation after a contraction caused by direct or indirect stimulation. Now, Biedermann found that when a galvanic current has been applied to the muscle in this tonic state there was a rapid relaxation around the anode with the making of the current and a moderate relaxation around the cathode at the breaking of the current. Apparently the making and the breaking of a galvanic current is capable of inhibiting the tonic state of the muscle. As the same results were also obtained in curarized muscles the experiment demonstrates that the muscle tissue itself is capable of responding with an inhibitory reaction. Highly interesting are the studies of Biedermann on the claw of the crawfish. When the abductor was divided and the nerve of the claw was stimulated it was found that a moderate stimulus caused a relaxation of the tonically contracted adductor, while a stronger stimulus caused its contraction; but when the adductor

was cut and the nerve stimulated, the abductor muscle contracted with a moderate and relaxed with a strong stimulus. When each muscle was separately connected with a lever graphically marking their movements, and the stimulation of the nerve was gradually increased, it was found that the first effect was that of contraction of the abductor; next came the relaxation of the adductor; then followed the contraction of the adductor, and about simultaneously with it the relaxation of the abductor. These experiments show that each muscle is provided with inhibitory and motor nerves but that in the abductor it is the motor nerve which is most sensitive while in the adductor it is the inhibitory nerve. The result is that a stimulus which is sufficient to contract the adductor keeps the abductor relaxed, and that a stimulus which causes the contraction of the abductor causes directly the relaxation of its antagonist. This is a beautiful example of Sherrington's reciprocal innervation. These experiments show that inhibitory effects may be discovered by the use of different intensities of stimulations. Another differentiating point is the time needed for the degeneration of nerves after they are cut. For instance, vasoconstrictors degenerate about four days after their section, while it takes ten days or more for the degeneration of vasodilators.

No doubt a great many more ways and means will be found to demonstrate the presence of inhibitory phenomena in the tissues and functions and the mode of their action in the various processes of life. But even as the matter stands today, with numerous well established facts at our disposal, we are entitled to insist upon the assumption that inhibition is an effective factor in all processes of life. We must keep in mind that the results as we have them today were attained in uphill work, that the attitude of many physiologists is generally inimical to admission of the presence of inhibitory phenomena, and that each new fact in this line had to struggle with various interpretations.

Before coming to the conclusion, I wish to quote here the most striking statement of Charles Bell made nearly a century ago. "The nerves have been considered so generally as instruments for stimulating the muscles, without thought of their acting in the opposite capacity, that some additional illustration may be necessary here.

Through the nerves is established the connection between the muscles, not only that connection by which muscles combine to one effort, but also that relation between the classes of muscles by which the one relaxes and the other contracts. I appended a weight to a tendon of an extensor muscle which gently strengthened it and drew out the muscle; and I found that the contraction of the opponent flexor was attended with a descent of the weight, which indicated the relaxation of the extensor. To establish this connection between two classes of muscles whether they be grouped near together, as in the limbs, or scattered widely as the muscles of respiration, there must be particular and appropriate nerves to form this double bond, to cause them to conspire in relaxation as well as to combine in contraction. If such a relaxation be established, through the distribution of the nerves between the muscles of the eyelids and the superior oblique muscles of the eyeball, the one will relax while the other contracts."

Permit me now to formulate the dualistic conception of the processes of animal life. Irritability means the property of living animal tissues to react with a change in their state to a proper stimulus. The change may be two-fold: it may consist in an excitation, in an increase of activity; or it may consist in an inhibition, in a greater or less decrease in activity. Tissues and organs in a state of inhibition are as much in a living state as when they are in a state of excitation or activity. In a state of activity living material is used up (catabolism). In a state of inhibition living material is replenished (anabolism). Each and every state of life of plain tissue or of complex functions is a resultant of the combination of the two antagonistic factors, excitation and inhibition. In a state of utmost rest the factor of inhibition prevails greatly. But there is still a sufficient remnant of the factor of excitation to permit the tissues or the functions to remain in a state of tonus, of dormant life. On the other hand, in a state of extreme excitation there is still a remnant of the factor of inhibition which thus prevents the excitation from consuming the living material which is indispensable to the life of the involved tissues.

Besides its indispensable value to the anabolic processes, inhibition is of importance to the prompt and proper activities of functions by elimination of simultaneous activities of elements directly

antagonistic to the success of that function.   It is also valuable to the economy of life by preventing the wasting of energies of living neighboring cells which are not needed for the performance of the specific function but are merely stimulated by irradiation of the exciting impulse from the chief focus.

THE CHEMICAL STRUCTURE OF CHONDRIDIN.*

BY P. A. LEVENE AND J. LÓPEZ-SUÁREZ.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Hebting,[1] working in Hofmeister's laboratory, has obtained on hydrolysis of chondroitin sulfuric acid a crystalline product which he named chondridin. Hebting recognized that the substance was related to chondrosin, but found that it differed from the latter in its elementary composition and its capacity to crystallize.

The structural representation of chondroitin sulfuric acid as formulated by Schmiedeberg, and later by Levene and La Forge, admits in the organic radical of the molecule the presence of only one derivative of chondrosin; namely, of its acetyl derivative. It therefore seemed probable that chondridin was not a primary decomposition product, but one formed from chondrosin through manipulation. This hypothesis was borne out by the present investigation. Hebting has found that the composition of chondridin differed from chondrosin by the presence in the molecule of a surplus of hydrogen and oxygen equivalent to 1 molecule of water.

A simple explanation of this difference may be given by assuming that chondridin consists of unchanged chondrosin containing a molecule of crystal water. On the other hand this assumption was scarcely adequate to explain the great difference in the capacity for crystallization of the two substances. More adequate seemed the assumption that chondridin was a lactone of chondrosin, crystallizing with crystal water. The substance analyzed by Hebting apparently

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* I regret that the article of Schmiedeberg (Schmiedeberg, O., *Arch. exp. Path. u. Pharmakol.*, 1920, lxxxvii, 47) had escaped my attention until after this work was prepared for publication. The present communication incidentally contains an answer to his criticism on my theory of the place of linkage of chondrosamine and of glucuronic acid. Other criticisms of Professor Schmiedeberg have been met in previous publications.—P. A. L.

[1] Hebting, J., *Biochem. Z.*, 1914, lxiii, 353.

contained 2 molecules of crystal water. The samples analyzed by us seemed to contain $2\frac{1}{2}$ molecules. The graphic representation of the anhydrous substance would then be as follows:

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H C OH
    |\
    | \
H C OH \
    |    O
  --C H  /
    |  /
    |/
  H C
    |    |        --O--         .
O H C — O — C — C — C — C — C — CH₂OH
    |    |   NH₂ H  H    OH
  --C       H  H  OH | H
    ‖
    O
```

NH₂ H   H   OH
H   H   OH
CH₂OH
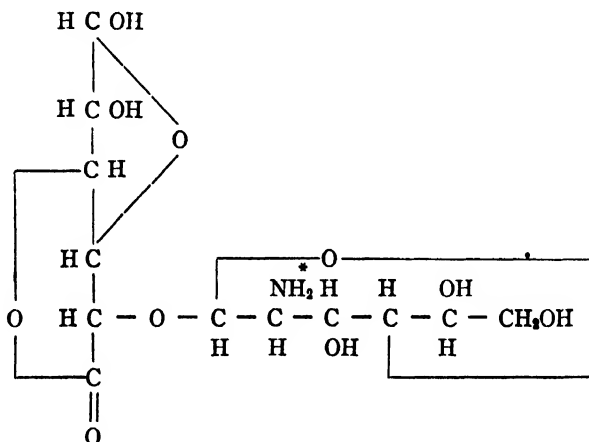
* Allocation of NH₂ arbitrary.

The elementary composition of this substance containing $2\frac{1}{2}$ molecules of crystal water is $C_{12}H_{19}NO_{10} + 2\frac{1}{2} H_2O$.

The experimental evidence to be reported in this communication seems to support the second hypothesis.

The lactone structure of the substance was suggested by the method of preparation and was confirmed by the result of the titration of the aqueous solution with alkali. When to a cold solution of the substance 0.1 N alkali is added rapidly, the solution reacts neutral after the addition of 1 drop of the alkali (alizarin being used as indicator). When to the aqueous solution of the substance the alkali was added in excess, and when the alkaline solution was titrated back after standing 18 hours, it was found that 0.1000 gm. of the substance neutralized 2.55 cc. of 0.1 N alkali. On this basis the molecular weight is calculated to 382, while the theory for $C_{12}H_{19}NO_{10} + 2\frac{1}{2} H_2O$ is 392. Thus, of the two assumptions that of the lactone structure of the substance seems the more acceptable.

The substance reduces Fehling's solution, has its primary amino group unsubstituted, and yields a quantity of furfurol which approximates the one required by theory for chondrosin.

The air-dry substance on drying under diminished pressure at the temperature of water vapor loses $1\frac{1}{2}$ molecules of crystal water. If the lactone structure of the substance is correct one has to accept that under the given conditions of drying the resulting substance still retains 1 molecule of crystal water.

EXPERIMENTAL PART.

The mode of preparation of the substance differed in its details from that of Hebting. The analytical data published by this author for chondridin seemed to agree with those required by theory for chondrosin oxalate. At the outset of the work it was planned to test this possibility. Chondrosin chlorohydrate was prepared following the conditions employed in the earlier work by Levene and La Forge. The chondrosin chlorohydrate obtained in this manner was freed from hydrochloric acid. To the aqueous solution of chondrosin a slight excess over one equivalent of oxalic acid was added and to the solution alcohol was added to opalescence. On standing there was no evidence of crystallization. However, when the solution was allowed to stand on a boiling water bath for an hour prior to the addition of alcohol, crystallization did take place. Alcohol was added to marked opalescence and the solution was allowed to remain on the water bath until it clarified. On scratching along the walls of the beaker a crystalline deposit soon began to form. The substance was recrystallized by dissolving in hot water, adding to the aqueous solution 99.5 per cent alcohol to opalescence, and boiling the solution until it clarified. After three or four recrystallizations the substance contained only traces of mineral impurities.

In later experiments the preparation of the substance was somewhat simplified; namely, no attempt was made to isolate the chondrosin hydrochloride before the digestion with oxalic acid. The procedure was as follows. Portions of 50.0 gm. of the barium salt of chondroitin sulfuric acid were hydrolyzed by heating for 1 hour on the water bath in a solution of 150 cc. of 20 per cent hydrochloric acid. From the product of reaction barium and hydrochloric acid were removed and the solution was concentrated to a small volume under diminished pressure. The subsequent treatment was as above described.

Properties of the Substance.—The lactone is a white crystalline powder. It does not melt, but contracts and turns dark at 200°C. The analysis of the substance was as follows:

1. 0.1176 gm. of the substance (No. 43) dried to constant weight at the temperature of water vapor and under diminished pressure lost 0.0078 gm.

2. 0.1130 gm. of another sample (No. 81) under the same conditions lost 0.0075 gm.

	Calculated for $C_{13}H_{21}NO_{11} + 1\frac{1}{2}H_2O$ per cent	Found. per cent
H_2O	7.20	6.63

1. 0.1098 gm. of the above substance gave on combustion 0.1626 gm. of CO_2 and 0.0580 gm. of H_2O.

0.1867 gm. of the substance used for Kjeldahl nitrogen estimation required for neutralization 5.3 cc. of 0.1 N acid.

For the amino estimation 0.050 gm. of the substance was dissolved in 5 cc. of water.

2 cc. of the solution in the Van Slyke micro apparatus gave 1.36 cc. of nitrogen gas at $T = 20°C.$ and $P = 764$ mm.

2. 0.1055 gm. of the substance gave 0.1564 gm. of CO_2 and 0.0568 gm. of H_2O.

	Calculated for $C_{13}H_{19}NO_{10} + H_2O$. per cent	Found. No. 1. per cent	No. 2. per cent
C	40.54	40.43.	40.11
H	5.96	5.98	5.92
N	3.94		3.97
NH_2 N	3.94		3.89

The rotation of the air-dry substance was as follows:

$$[\alpha]_D^{20} = \frac{+\,0.97 \times 100}{1.6 \times 1} = +\,60.6°$$

Titration of the Substance with Alkali.—0.1000 gm. of the substance was dissolved in 25 cc. of water and titrated with 0.1 N sodium hydroxide. Alizarin was used as indicator. After the addition of the first drop the solution reacted neutral. 0.1000 gm. of the substance was dissolved in 25 cc. of water. 15 cc. of 0.1 N alkali were added and the solution was allowed to stand over night. It required 12.45 cc. of 0.1 N acid to titrate the solution to neutral.

	Calculated for $C_{13}H_{19}NO_{10}+2\frac{1}{2}H_2O$.	Found.
Molecular weight	382.17	392

Furfurol Estimation.—0.2000 gm. of the substance was distilled in the usual way with hydrochloric acid having a specific gravity of 1.06. The yield of the phloroglucide was 0.0248, which corresponds to 0.00744 gm. of glucuronic acid.

	Calculated for $C_{13}H_{19}NO_{10}+2\frac{1}{2}H_2O$. *per cent*	Found. *per cent*
Glucuronic acid.....................................	50.77	37.2

Taking into consideration the limit of error of the method the result is not unsatisfactory.

ON A POSSIBLE ASYMMETRY OF ALIPHATIC DIAZO COMPOUNDS.

BY P. A. LEVENE AND L. A. MIKESKA.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

In 1915 Levene and La Forge made an observation which pointed to the formation of a diazo compound as an intermediary phase in the process of transformation of glucosaminic acid ethyl ester into anhydro-manonic ethyl ester.[1, 2] This observation brought forth the possibility of asymmetry in the α-carbon atom of the diazo compounds of the sugar acids. Since it was known that also esters of aliphatic α-amino-acids on treatment with nitrous acid were convertible into optically active oxy-acids, and since it was known that under certain conditions the treatment of the amino-acids resulted in the formation of aliphatic diazo esters, it seemed to the authors possible that also in the latter there existed an asymmetry. In 1916, Levene and Senior undertook to test experimentally the existence of optical isomerism in the aliphatic diazo compounds. The work was interrupted because of the conditions of war, Dr. Senior having accepted a commission in the Chemical Warfare Service. The work was resumed in September, 1920, by the present writers. In November there appeared a publication by Marvel and Noyes[3] on the subject of the present article. The experimental results reported by these writers were negative.

Our own work is not yet completed, but in view of the just mentioned publication we wish to record results which point favorably towards the possibility of asymmetry in these diazo compounds.

Dextro aspartic acid was converted into diazosuccinic acid ethyl ester. It was found possible to purify the ester by fractional distillation under a pressure varying from 0.08 to 0.12 mm.

[1] Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1915, xxi, 345.

[2] Levene, P. A., *J. Biol. Chem.*, 1918, xxxvi, 89.

[3] Marvel, C. S., and Noyes, W. A., *J. Am. Chem. Soc.*, 1920, xlii, 2259.

The analytical data of the samples are given in the following table.

	N	Impurity.	$[\alpha]_D^{20}$
	per cent	*per cent*	
Experiment 1.			
First fraction..............................	10.06	30.0	+1.27
Second "	13.68	5	+1.25
Third "	14.10	0	+1.34
Experiment 2.			
First fraction..............................			
Second "	13.64	2.5	
Third "	13 33	5	.
Second and third fractions redistilled.			
First fraction..............................	13.76	1.7	+1.27
Second "	13.64	2.5	
Experiment 3.			
First fraction..............................			
Second "	13.64	2.5	+1.00
Third "	14.20	0	+0.85

The theory for $C_8H_{11}O_4N_2$ requires N = 14.02 per cent.

The accuracy of the analytical method was tested on the crystalline diazosuccinic acid monoamide monoethyl ester and on diazoacetic acid ethyl ester.

The impurity of the highest optical activity that could be present in our material is *d*-malic diethyl ester. The optical rotation of this ester is $[\alpha]_D^{20} = +10.18$. It would require the presence of from 8 to 13 per cent of the latter compound if the rotation of our samples were brought about by impurities and not by the diazo compound. Impurities of such proportions could not have escaped detection by the analytical method employed in this work.

On hydrolysis of the diazo esters, optically active material was formed with a magnitude of rotation which did not differ essentially from that of the original material.

The details of the experiments will be reported in a subsequent communication. Further work is in progress.

d-RIBOHEXOSAMINIC ACIDS.

BY P. A. LEVENE AND E. P. CLARK.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

In previous publications[1-4] three pairs of epimeric hexosaminic acids were described. In that series the direction and numerical value of the optical rotation of the α-carbon atom were determined. On the basis of the direction of the rotation of the α-carbon atom all α-hexosaminic acids can be divided into two groups: the dextro and the levo acids.

On addition of prussic acid to a pentose two epimeric hexonic acids are obtained, one form as a rule predominating. On addition of prussic acid to an aminopentoside two epimeric α-hexosaminic acids are obtained, one form predominating. If a given pentose forms predominatingly a levo acid the corresponding aminopentoside also forms the levo-hexosaminic acid in excess over the epimer, and *vice versa*.

This seemed to suggest that in the levo-hexonic and in the levo-α-hexosaminic acids the configuration of the α-carbon atom is analogous, the position of the hydroxyl in one being the same as that of the amino group in the other. Furthermore, it was found that on deamination of the amino-acids the resulting oxy-acids were of the opposite sign. Fischer, on the basis of his extensive studies on Walden inversion, reached the conclusion that the presence of an adjoining carboxyl group is required for the occurrence of the inversion. If this rule holds good also for the α-amino sugar acids, then the observation just referred to offers additional evidence in favor of the assumption that in hexonic acids and in aminohexonic acids

[1] Levene, P. A., *J. Biol. Chem.*, 1918, xxxvi, 73.

[2] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1917, xxxi, 623.

[3] Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1915, xx, 433.

[4] Levene, P. A., *J. Biol. Chem.*, 1918, xxxvi, 89.

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of the same sign the configuration of the α-carbon atom is identical, the position of the hydroxyl in one being the same as that of the amino group in the other.

If the amino sugar or its lactone is deaminized and subsequently converted into the sugar acid, this, as a rule, is of the same sign as the α-aminohexonic acid corresponding to the sugar or the lactone. This observation also is in harmony with Fischer's observations on α-amino-acids. Also, as a rule, there was no inversion in the esters. Exceptions to this rule were observed in the esters of the α-amino-acids as well as in the lactones of the hexosaminic acids.

In the present communication the syntheses of two epimeric acids derived from ribose are reported. Thus the synthesis of the entire series of *d*-hexosaminic acids is completed. The observations made on this new pair agree in every way with the observations made on the other acids, namely:

1. The predominating acid is the levo acid, the same as on the addition of prussic acid to ribose.

2. On deamination of the acids, the change of sign occurs when nitrous acid acts on the acid and does not occur when it acts on the lactone.

· The following is a table of the direction of the rotation of the α-carbon atoms in the hexosaminic acid and in their products of deamination.

Acids	Rotation of the α-carbon.	Deamination product, α-carbon atom.
Chitosaminic	Levo.	Dextro.
Epichitosaminic	Dextro.	Levo.
Chondrosaminic	Levo.	Dextro.
Epichondrosaminic	Dextro.	Levo.
Dextro-xylohexosaminic	"	"
Levo-xylohexosaminic	Levo.	Dextro.
Dextro-ribohexosaminic	Dextro.	Levo.
Levo-ribohexosaminic	Levo. ·	Dextro.

If one then assumes that the hexosaminic acids and hexonic acids of the same sign have the α-carbon atom of an analogous configuration, then the configuration of the hexosaminic acids and their corresponding sugars are as follows:

A Chitosaminic   acid.....................Manonic   acid.
   (Glucosaminic acid)
   Epichitosaminic   "   ....Gluconic   "
B Dextro-xylohexosaminic "   ....Gulonic   "
   Levo-xylohexosaminic   "   ....Idonic   "
C Chondrosaminic   "   ....Talonic   "
   Epichondrosaminic   "   ....Galactonic "
D Dextro-ribohexosaminic "   ....Allonic   "
   Levo-ribohexosaminic   "   ....Altronic   "

Future work may prove that in hexonic and hexosaminic acids of the same sign the configuration of the α-carbon atom is not identical. The observations presented here will nevertheless retain their value, inasmuch as the configuration of all acids of this class will become clear as soon as the configuration of only one of them is definitely established.

There remains still to be mentioned the remarkable identity in the numerical value of the rotation of the α-carbon atom in the hexonic and hexosaminic acids.

The following is a table representing, on one hand, the numerical value of the α-carbon atom of the phenylhydrazides[2] of the hexonic acids and, on the other, of the corresponding hexosaminic acids.

Gluconic	14.25	Epichitosaminic	12.5
Manonic		Chitosaminic	
Gulonic	14.25	Dextro-xylohexosaminic	12.5
Idonic		Levo-xylohexosaminic	
Galactonic	8.25	Epichondrosaminic	12.5
Talonic		Chondrosaminic	
Allonic	20.8	Dextro-ribohexosaminic	19.12
Altronic		Levo-ribohexosaminic	

Only in the pair of galactonic and talonic acids is there noted a great difference in the numerical value of the rotation in the two series. This is probably due to the fact that talonic acid has not been obtained in pure form. This finding also shows that the superposition theory of optical rotation is only acceptable with certain reservations.

The present communication contains also improved directions for the preparation of ribose from yeast nucleic acid.

Preparation of d-Ribose.

The ribose used in these experiments was prepared from the purine nucleosides, guanosin and adenosin, obtained from yeast nucleic acid by ammonia hydrolysis. To prepare these nucleosides 400 gm. of yeast nucleic acid, suspended as a smooth paste in a hot solution of 320 cc. of concentrated ammonia and 1,680 cc. of water, were heated at 145° for 2 hours in an autoclave. After cooling, the solution was removed from the autoclave and placed in the refrigerator over night when a portion of the guanosin separated out. It was filtered off and treated as follows.

The crude guanosin was dissolved in a considerable quantity of boiling water, about 4 per cent solution, and made strongly acid to litmus with acetic acid, after which normal lead acetate was added until no more precipitate was formed. The precipitate was filtered from the boiling solution and the filtrate was then treated alternately with ammonia and basic lead acetate until a drop of either reagent gave no further precipitate. This precipitate was filtered off on a Buchner funnel, ground up in a mortar with water, and then filtered again.

After removing as much water as possible, it was ground up to a smooth paste with hot water, diluted to a considerable volume, made slightly acid with acetic acid, heated to the boiling point, and treated with hydrogen sulfide. When the lead was completely precipitated the solution with the lead sulfide was brought again to a boil and filtered. Upon cooling, guanosin crystallized out. This was filtered off and the mother liquors were evaporated to a small volume when a second crop was obtained.

The crude guanosin thus obtained was purified by repeating the above process with neutral and basic lead acetate, etc., and recrystallizing the resulting product from boiling water with a little charcoal.

To obtain the remaining guanosin and the adenosin, the filtrate from the original hydrolysis was made strongly acid with acetic

acid and treated first with normal lead acetate, then with basic lead acetate and ammonia exactly as given above for guanosin.

The filtrate from the treatment with hydrogen sulfide was concentrated to a rather heavy solution. During this operation more guanosin separates out from time to time. This is filtered off and purified as outlined above. The filtrate was diluted to about 1 liter and made acid to Congo red with sulfuric acid and then treated with a hot concentrated solution of picric acid until no further precipitate was formed. The precipitate was crude adenosin picrate. This was purified by recrystallizing from boiling water.

After the nucleosides were obtained in a pure condition they were hydrolyzed in an acid medium to obtain the sugar. For guanosin the following method was used. 100 gm. of guanosin were dissolved in 10 liters of boiling 0.1 N sulfuric acid and hydrolyzed by gentle boiling for 1 hour. Then to the hot solution an excess of silver sulfate suspended in a little water was added. The solution was allowed to stand over night, after which the guanine silver sulfate compound was filtered off and the clear solution freed from the excess of silver with hydrogen sulfide. The hydrogen sulfide was removed with a current of air and the sulfuric acid with chemically pure barium hydroxide. Great care had to be taken to get the solution exactly neutral to litmus and at no time was it allowed to become alkaline. It was best to add the barium hydroxide slowly in a rather dilute solution testing from time to time with litmus paper.

The barium sulfate was allowed to settle for a short time when most of the solution was drawn off from the precipitate. The remainder was filtered through a Buchner funnel and the combined solutions were then concentrated under reduced pressure at a low temperature to about 800 cc. when it was again made exactly neutral to litmus with dilute barium hydroxide water and filtered. The filtered solution was then concentrated under diminished pressure to a rather thick syrup and taken up in about 500 cc. of absolute alcohol. 300 cc. of ether were then slowly added with constant stirring. The precipitate formed was allowed to settle over night and then the solution was filtered. The filtrate was evaporated to a thick syrup as before and taken up with about 300 cc. of absolute alcohol and again evaporated. This evaporation with alcohol was

done three times to remove all water. The last time it was concentrated to about 60 cc., then warmed on a water bath, and removed as completely as possible from the flask by draining about 5 minutes. The residual syrup was then washed from the flask with 40 cc. of absolute alcohol in small portions and added to the main syrup, making the total volume 100 cc. Dry ether was then added until the syrup became turbid. It was then seeded and allowed to crystallize in a small desiccator. After 2 or 3 days the crystalline mass was broken up and filtered. The sugar was washed with a little absolute alcohol and ether mixture (2 parts alcohol to 1 part ether) then with ether and dried in a desiccator over sulfuric acid. By evaporating the mother liquor and adding ether as outlined above another crop was obtained. The yield is generally 82 to 83 per cent of the theory.

The ribose from the adenosin picrate was obtained as follows.

150 gm. of the recrystallized adenosin picrate were dissolved in 12 liters of boiling distilled water. When solution was complete 70 gm. of 95 per cent sulfuric acid, diluted in about 200 cc. of water, were cautiously added. The hydrolysis was effected by gentle boiling for 1 hour. The solution was then allowed to stand in the refrigerator over night when the adenine picrate crystallized out. This was filtered off and enough dilute barium hydroxide solution to neutralize 60 gm. of the sulfuric acid was slowly added with constant stirring.

The barium sulfate was allowed to settle and as much of the supernatant liquid drawn off as possible, the remainder was filtered from the barium sulfate, and the combined solutions were evaporated under reduced pressure to about 1 liter. This solution was then filtered and extracted with ether to remove the last traces of picric acid. When the extraction was completed the aqueous solution was diluted to about 4 liters and cautiously neutralized with barium hydroxide, then it was evaporated to about 800 cc. and again made exactly neutral to litmus with barium hydroxide. The further treatment was the same as in the preparation from guanosin.

The ribose remaining in the mother liquors from the second crop of sugar in all experiments was recovered by forming the *p*-bromophenylhydrazone. To this end an aliquot portion of the liquors was titrated and the calculated amount of *p*-bromophenylhydrazine

added. After a short time the hydrozone began to crystallize out. It was then filtered off and recrystallized from a small amount of absolute alcohol and decomposed in the ordinary way with benzaldehyde. This procedure, however, would hardly be expedient unless the mother liquors from a comparatively large amount of material were used, as relatively little sugar remains after the second crop.

The ribose was recrystallized by dissolving in distilled water, making a 10 per cent solution, and decolorizing with a little charcoal. It was then evaporated to a thick syrup, taken up several times in absolute alcohol, and evaporated so as to remove all the water, as outlined above, and then crystallized.

About 1.5 kilos of ribose have been made by this method with good and uniform results.

Preparation of Amino-d-Riboside.

This substance has been prepared and analyzed previously by Levene and La Forge.[3] It was found in the course of the present work that in order to obtain the maximum yield of the pure substance care must be exercised as to the dryness of the methyl alcohol and of the pentose. When either one of the substances is imperfectly dried the resulting product contains a certain proportion of the dipentose derivative, of the structure:

$$
\begin{array}{cccc}
\text{H C} & \!\!\!\text{---NH---} & \text{C H} \\
\text{HCOH} & & \text{HCOH} \\
& \text{O} & & \text{O} \\
\text{HCOH} & & \text{HCOH} \\
\text{H C} & & \text{H C} \\
\text{C H}_2\text{OH} & & \text{C H}_2\text{OH}
\end{array}
$$
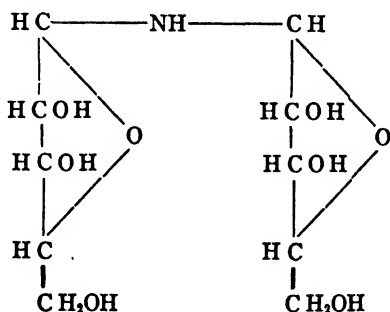
Under favorable conditions the yield of the amino derivative is between 90 and 95 per cent of the theory.

Preparation of the Dextro- and Levo-d-Ribohexosaminic Acids.

As in the preparation of other α-hexosaminic acids, so also in the preparation of these two acids the yield depends on the tempera-

ture of the reaction, on the proportion of prussic acid, and on the duration of the reaction between prussic acid and the aminopentoside. Optimal yields of 60 to 65 per cent of the employed ribose were obtained only at the very end of the work.

The optimal conditions are as follows. 20.0 gm. of the aminopentoside dried under diminished pressure at room temperature were dissolved in 40.0 cc. of water and 30 cc. of crude freshly prepared prussic acid (of 80 per cent strength) and 5 cc. of ammonia water were added. The solution was rapidly warmed to 30°C. and kept at that temperature for 15 minutes. The temperature should be watched very carefully. The further treatment is similar to that in the preparation of other *d*-hexosaminic acids.

When only 20 cc. of prussic acid were added instead of 30.0 cc., the yield of acids did not exceed 40 per cent and often did not reach that value.

The crystallization of the mixed acid is brought about in the following way. The product of reaction prepared for crystallization is made up with water to 25 cc. and to this hot methyl alcohol is added to marked opalescence. The solution is then placed on a water bath and stirred with a glass rod until the appearance of a crystalline deposit.

The optical rotation of the mixed acids was as follows:

$$[\alpha]_D^{20} = \frac{-0.16 \times 100}{1 \times 2} = -8.0°$$

Separation of the Two Acids.

A. Levo-d-Ribohexosaminic Acid.

Levo-*d*-ribohexosaminic acid is the more insoluble form and is prepared without much difficulty. Two experiments are here reported.

Experiment 1.—57.0 gm. of the mixed acids were dissolved in 70.0 cc. of boiling water and allowed to crystallize over night. The yield of the crystalline deposit was 22.0 gm. This substance had the following optical rotation (No. 6381$\frac{1}{2}$):

$$[\alpha]_D^{20} = \frac{-0.52 \times 100}{1 \times 2} = -26.0°$$

This rotation indicates that the substance was the pure levo form. On recrystallization out of water two fractions were obtained (No. 640, 641½) each having the same rotation, and as will be shown later the substance prepared from the pure crystalline lactone possesses the same optical rotation.

Experiment 2.—142.0 gm. of the mixed acids were dissolved in 300 cc. of boiling water. 35.0 gm. of the levo form crystallized over night. The rotation of the substance was as follows (No. 88?):

$$[\alpha]_D^{20} = \frac{-0.52 \times 100}{1 \times 2} = -26.0°$$

On further recrystallization the rotation of the substance did not change.

Properties of the Levo-d-Ribohexosaminic Acid.—The substance crystallizes in thin plates resembling those of cholesterol. It is soluble in water and insoluble in the usual organic solvents. The melting point of the substance is M. P. = 212°C. (uncorrected) with decomposition.

Lactone Hydrochloride of the Levo-d-Ribohexosaminic Acid.—12.0 gm. of the acid carefully dried and pulverized were suspended in 600 cc. of alcohol (99.5 per cent) and dry hydrochloric acid gas was passed for 15 minutes. The acid dissolved almost immediately. The solution was concentrated under diminished pressure at ordinary temperature until crystallization took place in the distillation flask. The contents were then transferred to an evaporating dish and allowed to stand over night in a desiccator over sulfuric acid. The yield of the lactone was 12.0 gm. The melting point of the substance was M. P. = 188°C. (uncorrected). It had the following composition (No. 24?):

0.1076 gm. of the substance gave 0.1304 gm. of CO_2 and 0.0592 gm. of H_2O.

0.2000 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 8.74 cc. of 0.1 N acid.

0.2000 gm. of the substance required 9 cc. of 0.1 N $AgNO_3$ to titrate its hydrochloric acid.

	Calculated for $C_6H_{12}NO_6Cl$. per cent	Found. per cent
C	33.71	33.05
H	5.67	6.17
N	6.55	6.12
Cl	16.25	15.96

The substance still contained 0.46 per cent of mineral impurity. The peculiarity of this lactone was that by the Van Slyke method only 4.71 per cent of N was obtained, even when the reaction was allowed to proceed for 30 minutes.

The rotation of the substance was the following:

$$[\alpha]_D^{20} = \frac{-0.22 \times 100}{1 \times 2} = -11.0°$$

Conversion of the Lactone into Free Acid.—5.0 gm. of the lactone hydrochloride were dissolved in 50 cc. of water, the solution was treated with an excess of barium hydroxide, and allowed to stand over night. The barium and hydrochloric acid were removed from the solution and the free acid was crystallized on concentration of the aqueous solution. For analysis it was recrystallized out of water on addition of a little alcohol. After three recrystallizations the substance was analyzed. The melting point was M. P. = 212°C. uncorrected). It analyzed as follows:

0.1010 gm. of the substance gave 0.1364 gm. of CO_2 and 0.1010 gm. of H_2O.
0.1990 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 10.10 cc. of 0.1 N acid.

	Calculated for $C_6H_{13}NO_5$. per cent	Found per cent
C	36.92	36.83
H	6.66	7.04
N	7.18	7.10

The rotation of the substance was the following:

$$[\alpha]_D^{20} = \frac{-0.52 \times 100}{1 \times 2} = -26.0°$$

B. *Dextro-d-Ribohexosaminic Acid.*

The separation of the dextro form was found more difficult than that of its epimer, and was accompanied with considerable loss of material. The procedure finally adopted was as follows. All fractions with the optical rotation above 0.0° were combined, dissolved in about 5 to 8 volumes of hot water, and methyl alcohol was added to initial opalescence. The solution was then placed on a boiling water bath and there allowed to crystallize. The crystalline deposit

was filtered off while the mother liquor was still hot. The operation was repeated until a constant rotation was obtained. This was found to be as follows:

$$[\alpha]_D = \frac{+ \ 0.25 \times 100}{1 \times 2} = + \ 12.5°$$

After three recrystallizations the rotation remained unchanged. As will be seen later the substance obtained from the lactone possessed the same optical activity. The substance had the melting point M. P. = 186°C. (uncorrected) and analyzed as follows:

0.1057 gm. of the substance gave 0.1422 gm. of CO_2 and 0.0626 gm. of H_2O.
0.2000 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 10.20 cc. of 0.1 N acid.

	Calculated for $C_6H_{13}NO_5$. per cent	Found. per cent
C..	36.92	36.70
H..	6.66	6.63
N..	7.18	7.14

Lactone Hydrochloride of Dextro-d-Ribohexosaminic Acid.—5.0 gm. of the acid carefully dried under diminished pressure at 50°C. and pulverized were suspended in 300 cc. of absolute alcohol (99.5 per cent) and dry hydrochloric acid gas was passed through the alcohol. Solution was accomplished almost immediately. The gas was passed 7 minutes. The solution was then concentrated under diminished pressure until a considerable sediment began to form in the distilling flask. The material was then transferred to an Erlenmeyer flask. The sediment on standing increased in volume, but was found to be amorphous. On heating, however, the sediment redissolved and on prolonged standing on the water bath with stirring a sediment of heavy crystals of the lactone settled out.

The substance had the melting point M. P. = 150°C. (uncorrected) and analyzed as follows:

0.0977 gm. of the substance gave 0.1212 gm. of CO_2 and 0.0546 gm. of H_2O.
0.1872 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 8.85 cc. of 0.1 N acid.

	Calculated for $C_6H_{12}NO_5Cl$. per cent	Found. per cent
C	33.71	33.83
H	5.67	6.25
N	6.55	6.62

The rotation of the substance was as follows:

$$[\alpha]_D^{20} = \frac{+0.43 \times 100}{1 \times 2} = +21.5°$$

Conversion of the Lactone into the Dextro-d-Ribohexosaminic Acid.—
Three grains of the lactone hydrochloride were dissolved in 25 cc.
of water. The solution was rendered alkaline by means of barium
oxide and allowed to stand over night. The barium and the hydro-
chloric acid were then removed and the remaining aqueous solution
was concentrated to a small volume. To the concentrated solution
alcohol was added to opalescence, and the solution was allowed to
digest on a boiling water bath until a heavy crystalline deposit formed.
The yield of this substance was 2.0 gm.

The melting point of the substance was M. P. = 186°C. (uncor-
rected). The rotation was as follows:

$$[\alpha]_D^{20} = \frac{+0.25 \times 100}{1 \times 2} = +.12.5°$$

Thus the melting point and the optical rotation of the substance
purified through conversion into its lactone and reconversion of this
into the acid remained identical with those of the original material.

Dibenzal-Dextro-d-Ribohexosaminic Ethyl Ester Hydrochloride.

The dextro form differed from its epimer in that it formed the
above compound under the same condition in which the levo form
gave rise to its lactone hydrochloride. 2.0 gm. of the carefully
dried and pulverized acid were suspended in 20.0 cc. of absolute
alcohol (99.5 per cent) to which 2.0 cc. of redistilled benzaldehyde
were added, and dry hydrochloric acid gas was passed through the
solution. The acid dissolved rapidly, but the treatment with acid
was continued for 7 minutes. The slightly turbid solution was
allowed to stand over night. A crystalline deposit consisting micro-
scopically of long needles was formed. It was filtered off, washed

with alcohol and ether, dried, and analyzed. The substance (No. 232) had a melting point M. P. = 221°C. (uncorrected) and the following composition:

0.1069 gm. of the substance gave 0.3270 gm. of CO_2 and 0.0602 gm. of H_2O.

0.1978 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 4.60 cc. of 0.1 N acid.

	Calculated for $C_{22}H_{26}NO_8Cl$. *per cent*	Found. *per cent*
C	60.65	60.47
H	6.34	6.31
N	3.20	3.25

The rotation of the substance dissolved in methyl alcohol was the following:

$$[\alpha]_D^{20} = \frac{-0.26 \times 100}{1 \times 1} = -26.0°$$

From the mother liquor of the dibenzal derivative on standing a second crop of crystals formed, which once recrystallized had the composition and the physical properties of the lactone hydrochloride of the dextro-*d*-ribohexosaminic acid. This observation is important inasmuch as it offers additional evidence of the purity of the dextro-*d*-ribohexosaminic acid.

The substance analyzed as follows:

0.0942 gm. of the substance gave 0.1174 gm. of CO_2 and 0.0512 gm. of H_2O.

0.0918 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 4.5 cc. of 0.1 N acid.

	Calculated for $C_6H_{12}NO_6Cl$. *per cent*	Found *per cent*
C	33.71	33.99
H	5.67	6.08
N	6.55	6.86

The optical rotation of the substance in 2.5 per cent hydrochloric acid was

$$[\alpha]_D^{20} = \frac{+0.43 \times 100}{1 \times 2} = +21.5°$$

Oxidation of the d-Ribohexosaminic Acids with Nitric Acid.

Levo-d-ribohexosaminic acid on oxidation with nitric acid gave rise to α, α-anhydroallomucic acid. Two 5.0 gm. portions of the acid were dissolved each in 35 cc. of water; to the solution 15 cc. of 10

per cent hydrochloric acid and 5.0 gm. of silver nitrite were added. The mixture was allowed to react over night. Twice during the reaction 2.0 gm. portions of the nitrite and 2 cc. of hydrochloric acid were added. The reaction product was freed from excess of silver and the filtrate reduced to a volume of 75 cc. by distillation under diminished pressure at 40–50°C. of the water bath. To this solution 50 cc. of nitric acid were added and the resulting solution was heated over flame for 20 minutes and then rapidly concentrated to a thick mass on a water bath. The thick residue was dissolved in 10 cc. of a solution consisting of equal parts of water and concentrated nitric acid, then evaporated once with water to remove nitric acid. The final residue was then converted into the calcium salt of α, α-anhydroallomucic acid. For final analysis the salt was suspended in water and its calcium removed by boiling in hot water containing a slight excess over the required amount of oxalic acid.

The calcium salt of α, α-anhydroallomucic differs from that of anhydrotalomucic, first by being optically inactive and second by the difference in behavior on heating. Both salts crystallize with 3 molecules of crystal water. Heated under diminished pressure at the temperature of xylene vapors anhydroallomucic salt loses all its·3 molecules of crystal water, whereas the corresponding anhydrotalomucic salt loses only 2 retaining the third one.

The Ca salt of anhydroallomucic acid analyzed as follows:

0.1228 gm. of the salt on drying lost 0.0244 gm. in weight.

	Calculated for $C_6H_6O_7Ca + 3 H_2O$. *per cent*	Found. *per cent*
H_2O	19.02	19.87

0.0984 gm. of the substance gave on combustion 0.11 gm. of CO_2, 0.0270 gm. of H_2O, and 0.0984 gm. of CaO.

	Calculated for $C_6H_6O_7Ca$. *per cent*	Found. *per cent*
C...	31.30	30.99
H...	2.61	3.07
CaO..	24.35	25.00

Dextro-d-ribohexosaminic acid on oxidation gave rise to α, α-anhydrotalomucic acid. 5.0 gm. of the substance were deaminized in the same manner as the levo form. For oxidation the solution was

brought to 60 cc., to which an equal volume of nitric acid was added. The resulting solution was heated over free flame for 13 minutes. The product of reaction was transferred to clock glasses and rapidly concentrated nearly to dryness. The residue was reoxidized once more with a solution of equal parts of water and nitric acid, and finally once evaporated with water to remove nitric acid. The residue was then converted into Ca salt. The yield was 2.8 gm. For purification the salt was reconverted into free acid by means of oxalic acid and reconverted into the Ca salt. The pure salt crystallized partly in plates and partly in prismatic needles. Heated under diminished pressure at temperature of xylene vapors it lost 2 molecules of water. The salt was levorotary.

It analyzed as follows:

0.1216 gm. of the substance on drying lost 0.0160 gm. in weight.

	Calculated for $C_6H_8O_8Ca + H_2O$. per cent	Found per cent
H_2O	12.68	13.16

0.1056 gm. of the substance gave 0.1120 gm. of CO_2, 0.0302 gm. of H_2O, and 0.0242 gm. of CaO.

	Calculated for $C_6H_6O_7Ca + H_2O$. per cent	Found. per cent
C	29.03	28.98
H	3.22	3.20
CaO	22.58	22.92

The optical rotation of the substance was the following:

$$[\alpha]_D^{20} = \frac{-0.18 \times 100}{1 \times 2} = -9.0°$$

LECITHIN.

III. FATTY ACIDS OF LECITHIN OF THE EGG YOLK.

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Many considerations call for a reinvestigation of lecithins in regard to the character of their fatty acids.

The two earliest investigations on the fatty acids of lecithin gave occasion for the question of the existence of several lecithins varying from one another in the character of the fatty acids contained in their structure. Variations were claimed both on the point of the saturated and unsaturated acids. Each one of these points merits special discussion.

The first time an unsaturated acid was separated by Strecker[1] it was assumed by him to be oleic acid. The data on which the assumption was based could not be considered sufficient from a modern point of view. Subsequent workers, including Thudichum,[2] all referred to the unsaturated acid as oleic, on evidence of the same nature as that of Strecker. A very careful investigation on the unsaturated fatty acids of lecithin was published by Cousin,[3] who separated and conclusively identified oleic and linolic acids. More modern investigators following Cousin have added little to the results obtained by him. Stern and Thierfelder,[4] Baskoff,[5] Rollett,[6] and more recently Cruickshank[7] have brought forth evidence confirmatory to that of Cousin.

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[1] Strecker, A., *Ann. Chem.*, 1868, cxlviii, 77.

[2] Thudichum, J. L. W., A treatise on the chemical constitution of the brain, London, 1884.

[3] Cousin, H., *Compt. rend. Acad.*, 1903, cxxxvii, 68; *J. pharm. chim.*, 1906, xxiii, series 6, 225.

[4] Stern, M., and Thierfelder, H., *Z. physiol. Chem.*, 1907, liii, 370.

[5] Baskoff, A., *Z. physiol. Chem.*, 1909, lxi, 426.

[6] Rollett, A., *Z. physiol. Chem.*, 1909, lxi, 210.

[7] Cruickshank, J., *J. Path. and Bact.*, 1913–14, xviii, 428.

As regards the saturated fatty acids, Diacanow[8] was the first to separate one in pure form. It analyzed for stearic acid. All subsequent workers have found that the saturated acid contained also, besides stearic, a lower fatty acid. Some isolated an acid having the composition of palmitic (Strecker). No investigator has identified both of them in pure form, but the mass of evidence was in favor of the assumption of the presence of palmitic and stearic acids in the so-called lecithin.

Recently (1912), a very important contribution was made by Delezenne and Fourneau.[9] By the action of cobra venom on commercial lecithin they obtained an intermediate product of lecithin degradation. The substance contained in its molecule all the components of lecithin save the unsaturated acid. The product was crystalline. From this product they isolated an acid which analyzed correctly for palmitic. The melting point of their acid was 59–60°C., and that of pure palmitic, 64–65°C. They did not determine the molecular weight of their acid. It is, however, evident that their acid was, in the main, palmitic and might have contained only traces of impurity. The finding of Delezenne and Fourneau is of great importance as it reopened the question of the existence of more than one lecithin.

Indeed, the observations of all previous writers, granting that they were correct, could be equally well explained by one of the two alternative assumptions. First, that there existed several lecithins. Second, that lecithin contained only oleic and palmitic acid in its molecule, and that the linolic and stearic acids originated in the cephalin present in all crude lecithins. Cephalin, as is now known, contains linolic and stearic acids.

However, a pure lecithin free from cephalin was recently analyzed by Levene and Ingvaldsen.[10] This substance was derived from liver. The saturated acid contained in it was stearic and the unsaturated acid was not oleic but what seemed to be a homologue of linolic. Thus again the question of the existence of more

[8] Diacanow, C., *Centr. med. Wissensch.*, 1868, vi, 2.

[9] Delezenne, G., and Fourneau, E., *Bull. Soc. chim.*, 1914, iv, series 15, 421.

[10] Levene, P. A., and Ingvaldsen, T., *J. Biol. Chem.*, 1920, xliii, 361.

than one lecithin was forced to the front. All these considerations led to the reinvestigation of the problem of the fatty acids of lecithins freed from all traces of cephalin. The present work is a report on the lecithin of the egg yolk. Reports on the work on lecithin of other organs will be communicated later.

Lecithin of the egg yolk free from cephalin was found to contain only one unsaturated acid, namely oleic, and two saturated acids; namely, palmitic and stearic. The bulk of the material employed for this work was the cadmium chloride salt.

The oleic acid was identified by the iodine number and by the analysis of the hydrogenated product. The saturated acids were identified by their elementary composition, by their melting points, and by their molecular weights.

Furthermore, it was found that saturated and unsaturated acids were present in lecithin in equimolecular proportions. All these facts point towards the assumption of the existence of more than one lecithin in egg yolk.

An objection to these conclusions might be found in the assumption that the cadmium chloride product contained impurities which were not cephalin. To meet this objection hydrolyses were made on a smaller sample of dihydrolecithin prepared from the cadmium salt free from cephalin, and also on a smaller sample of free lecithin prepared from the same material. The results obtained from these experiments are confirmatory of those based on the analysis of the cadmium chloride derivative.

EXPERIMENTAL.

Preparation of Lecithin Cadmium Chloride from Acetone Extract of Egg Yolk.

The cadmium chloride salt of lecithin obtained from the acetone-soluble fraction of egg powder was used in this investigation as the source of amino-free lecithin. The following procedure has been found satisfactory. After exhaustively extracting egg powder with acetone, the extract is concentrated to a small bulk and the residual syrup is allowed to stand at 0°C. for at least 24 hours. During this period the higher melting fats separate in a crystalline condition and

are easily removed by filtration under suction, if the temperature is kept below 10°C.   Such fat contains a relatively constant quantity of lecithin, which is practically amino-free and forms, therefore, the best source of lecithin, uncontaminated by cephalin.

The cake of fat is melted on the water bath with two or three volumes of alcohol, the liquids are thoroughly mixed, and the fat is again allowed to crystallize at 0°.   After filtration the mother liquor is concentrated under diminished pressure to half its original volume and an alcoholic solution of cadmium chloride is added until no further precipitation occurs.   The cadmium chloride salt thus obtained is free from unsaturated oils and is therefore almost white in color, with no tendency to darken on exposure to air.

We have found the following method very efficient in reducing the amino content and improving the physical properties of any cadmium chloride salt of lecithin.   With salts obtained from the fat cake, one such purification is sufficient to yield amino-free material.

The pulverized salt is suspended in approximately its own volume of toluene.   If the material contains any moisture, a clear solution occurs immediately or on slight warming; if a colloidal suspension is formed, the addition of a few drops of water results in its immediate dissolution.   An opalescent effect indicates the presence of finely divided cerebrosides.   These may be entirely removed by centrifugalization.   The clear toluene solution is then poured into four volumes of ether containing 1 per cent of water. Precipitation occurs within a few minutes, if thoroughly cold ether is used.   The precipitate is separated by centrifuging, well washed with ether, and finally, in order to remove the toluene as completely as possible, suspended in acetone and filtered.

A sample of material prepared in this manner analyzed as follows:

No. 240. 2 gm. of substance were hydrolyzed with HCl, neutralized, and concentrated to 10 cc.

5 cc. of this solution required for Kjeldahl determination 3.16 cc. of 0.1 N HCl equivalent to 0.00302 gm. of N.

2 cc. of this solution for Van Slyke determination gave 0.03 cc. of N at $T = 21°$, $P = 766$ equivalent to 0.000017 gm. of N.

$$\cdot \; \frac{NH_2N}{Total\ N} = \frac{1.4}{100}$$

0.1942 gm. of substance used for Kjeldahl determination required 1.96 cc. of 0.1 N HCl.

0.2913 gm. of substance gave 0.0322 gm. of $Mg_2P_2O_7$.

	Calculated for $C_{44}H_{46}O_9NPCdCl_2$. per cent	Found No. 240. per cent
N	1.43	1.41
P	3.18	3.08

From 25 pounds of egg yolk powder, 80 to 100 gm. of such material may be obtained, depending upon the extent to which the acetone extract is concentrated before filtration. Great variation has been experienced in the yield of the lipoids extracted from different samples of egg yolk powder.

Good material may also be obtained from the acetone filtrate from the fat cake, although with greater difficulty. From 25 pounds of lipoid-rich egg yolk powder an additional 250 gm. of amino-free cadmium chloride salt may be obtained by the following procedure. The filtrate is treated with an alcoholic solution of cadmium chloride until no further precipitation occurs. Several volumes of warm acetone are added to the mixture, and the supernatant liquid is decanted as soon as the precipitate has settled. By allowing the precipitate to stand too long, the oil, which is originally suspended in the acetone and easily decanted, settles out with the cadmium salt, whereby the difficulty of effective separation is greatly enhanced. Four or five repetitions of this process bring the cadmium salt to a condition in which it is easily filtered. The amino content of the material at this state of purity varies from 15 to 25 per cent.

Several steps have been employed to effect its further purification, each one accomplishing a special end. These have been selected with a view to their efficiency; the first of removing fats and oils, the second of reducing the amino content, and the third of removing the final traces of cerebrosides and saturated lipoids.

1. The cadmium salt is suspended in warm ether. To this, water is added until the suspended material is dissolved. The solution is then allowed to stand at 0°C. for 24 hours, and the salt separates in flocculent form easily filterable by suction. If it is desired to dry the product, subsequent suspension in acetone and refiltration gave a precipitate more easily dried and handled than that separating directly from ether. The amino content of this material is about 7 to 10 per cent.

2. This material is then subjected to the toluene ether purification as described above; its amino content is thereby reduced approximately 50 per cent. On suspension in acetone a white or very pale, yellowish white waxy precipitate is formed which does not change color on drying and which is easily pulverized, having lost any sticky or gummy character.

3. The dry powder is suspended in ether, dissolved by the addition of water, and precipitated by adding the ethereal solution to several volumes of alcohol. If a Van Slyke determination still indicates the presence of amino nitrogen-containing material, the substance may be again subjected to any of these processes until the cadmium salt is amino-free.

Of such a sample the following is a typical analysis. It contained no amino nitrogen.

No. 18. 0.200 gm. of substance required for Kjeldahl estimation 2.10 cc. of 0.1 N HCl equivalent to 0.00294 gm. of N.

0.300 gm. of substance gave 0.0330 gm. of $Mg_2P_2O_7$.

	Calculated for $C_{44}H_{86}O_9NPCdCl_2$. per cent	Found No. 18. per cent
N	1.46	1.47
P	3.14	3.07

There was no difference apparent between the fatty acids isolated from the two fractions.

Preparation of Lecithin Cadmium Chloride from Ethereal Extract of Egg Yolk.

Egg powder which had been exhaustively extracted with acetone was further extracted with ether. This extract contains not only the unsaturated but also the saturated lipoids. These are removed in the following manner. The extract is reduced to a small volume by distilling off the greater part of the ether. The residue is precipitated by acetone. The precipitate thus formed is dissolved in ether and allowed to stand at 0°C. for 24 hours. The cerebrosides and sphingomyelin settle out in the form of a white precipitate. This process is repeated until no further precipitate is deposited from ether on standing. The material which is precipitated by acetone from the clear ethereal solution contains the so called "cephalin," pure cephalin, and lecithin. The lecithin is contained in the fraction soluble in alcohol. This is obtained in the following way. The

material is taken up in ether and alcohol is added until a definite precipitation occurs. After several repetitions no further solution occurs. The decanted alcoholic liquors are concentrated to a smaller bulk under diminished pressure. The lecithin is then precipitated as the cadmium chloride salt. Purification of the salt is continued according to the methods described in the preceding section, until no amino nitrogen is found in the hydrolyzed material by a Van Slyke estimation.

Isolation of the Fatty Acids.

Hydrolysis was effected by boiling the cadmium chloride salt for 10 hours with ten parts of 10 per cent hydrochloric acid. The crude mixture of saturated and unsaturated fatty acids was filtered off, on cooling. The yield of the crude acids was invariably 95 to 98 per cent of that anticipated by the theory.

They consisted apparently of equal parts of saturated and unsaturated acids, as is seen from the following experiment. The mixed acids transformed directly into lead salts yielded 46 gm. of total lead salts. Of these, 25 gm. were insoluble in ether. In a second experiment, in which the separation of the acids was effected by recrystallization from acetone, as described below, 8 gm. of saturated acids were obtained, as compared with a yield of 7.5 gm. of unsaturated acids.

Furthermore, the crude mixture of fatty acids gave an iodine number of 50.2. Since the unsaturated acid, as will be shown below, is oleic, this value again points towards the presence in the lecithin molecule of equimolecular proportions of saturated and unsaturated acids.

A. Fatty Acids of the Lecithin Prepared from the Acetone Fraction. Acids from Dihydrolecithin Prepared from This Fraction.

A sample of hydrolecithin was prepared from a cadmium salt of lecithin free from amino nitrogen. The reduction was accomplished by Paal's method. The mixed fatty acids obtained from this sample gave the following data:

All samples were dried by fusion on an electric hot plate, and to insure absolute freedom from moisture in the material used for combustion, were remelted under diminished pressure at the temperature of xylene vapor until constant weight was attained.

In all cases the melting points recorded in this paper are corrected and were taken at such a rate that the time interval per degree rise in temperature was 6 seconds. The molecular weights were calculated by the titration of approximately 1 gm. of acid, dissolved in 10 cc. of toluene and 25 cc. of methyl alcohol (neutral to phenolphthalein), with 0.5 N NaOH, using phenolphthalein as an indicator.

They melted at 57–58°, gave a titration value corresponding to a molecular weight of 273, and the following analysis:

0.1004 gm. of substance, dried by fusion, gave on combustion 0.1118 gm. of H_2O and 0.2780 gm. of CO_2.

1.049 gm. of substance, dried by fusion, required for neutralization 7.67 cc. of 0.5 N NaOH, corresponding to a molecular weight of 273.

Sample No.	Analysis.		Molecular weight.	Melting point.
	H	C		
	per cent	*per cent*		°C.
132	12.46	75 51	273	57–58

Saturated Acids Isolated from Lecithin Prepared from This Fraction.

The cake of mixed fatty acids was freed from water-soluble impurities by melting on the water bath and collecting the acids with a little benzene. On cooling, the cake was again readily separable. It was then dissolved in a small quantity of acetone, from which the saturated fatty acid crystallized almost quantitatively on cooling to −10°C. After filtration and thorough washing the separation of saturated from unsaturated acid was practically complete.

An additional recrystallization of the saturated acids from acetone completed the first crude separation of the saturated from the unsaturated acids. The saturated acids were then transformed into their lead salts, which were thoroughly extracted with boiling ether in order to remove all traces of unsaturated salts. By decomposition

with hydrogen sulfide, the acids were regained. The analysis of several samples follows.

No. 76. 0.1000 gm. of substance, dried by fusion, gave on combustion 0.1137 gm. of H_2O and 0.2760 gm. of CO_2.

1.0082 gm. of substance, dried by fusion, required for neutralization 7.57 cc. of 0.5 N NaOH corresponding to a molecular weight of 266.

No. 452. 0.0992 gm. of substance, dried by fusion, gave on combustion 0.1106 gm. of H_2O and 0.2730 gm. of CO_2.

1 gm. required for neutralization 7.44 cc. of 0.5 N NaOH corresponding to a molecular weight of 268.3.

No. 317. 0.1002 gm. of substance, dried by fusion, gave on combustion 0.1144 gm. of H_2O and 0.2757 gm. of CO_2.

Sample.	Analysis.		Molecular weight.	Melting point.
	H	C		
	per cent	per cent		°C.
Calculated for $C_{16}H_{32}O_2$ (palmitic acid)......	12.58	74.93	256	64–65
Calculated for $C_{18}H_{36}O_2$ (stearic acid).......	12.72	75.98	284	70–71
Found No. 76.............	12.72	75.26	266	58–59
" " 452....,....................	12.48	75.05	268	58–59
" " 317.....................	12.77	75.03	264	61

Fractional crystallization from acetone, ether, or alcohol caused no decided change, nor was repeated preparation of the lead salt with subsequent decomposition effective in fundamentally altering the analytical data.

With acetic anhydride, however, we separated a fraction which corresponded to palmitic acid. The mixed acids were dissolved in ten parts of warm acetic anhydride. A substance crystallized on cooling, which after recrystallization consisted, according to the indications given by its molecular weight and analysis, of a mixture of acid and anhydride. Purification by conversion into the sodium salt and extraction of the latter with acetone with subsequent decomposition gave an acid of the following analysis:

No. 131. 1.0020 gm. of substance, dried by fusion, gave on combustion 0.1177 gm. of H_2O and 0.2755 gm. of CO_2.

1.0887 gm. of substance, dried by fusion, required for neutralization 8.33 cc. of 0.5 N NaOH corresponding to a molecular weight of 257.

Substance.	Analysis.		Molecular weight.	Melting point.
	H	C		
	per cent	*per cent*		*°C.*
Calculated for $C_{16}H_{32}O_2$ (palmitic acid)......	12.58	74.93	256	64–65
Found No. 131............................	13.14	74.98	257	64–65

The isolation of both constituents of this mixture was effected by the fractional distillation of the methyl esters under diminished pressure. The mixture of methyl esters was prepared by the usual method of esterification with methyl alcohol and sulfuric acid.

The mixed esters, No. 126, the saponification value of which corresponded to a molecular weight of the acid equal to 266, were distilled at a pressure of 1.6 mm. into four fractions. These fractions were then saponified. The molecular weights, melting points, and analyses of their respective acids are recorded below under the corresponding numbers.

Sample.	Boiling point of ester.	Molecular weight of acid.	Melting point of acid.	Analysis of acid.	
				H	C
	°C.		*°C.*	*per cent*	*per cent*
Mixed esters, No. 126		266	58–59	13.06	75.34
First fraction, No. 133...........	145–159	255	61–62	12.96	74.70
Second " " 134	155–170	260	61–62	12.83	75.49
Third " " 135	160–178	264	61–62	13.20	75 07
Residue, " 136...........		278	72–72.5	12.98	75.56

No. 126.   1.004 gm. of substance, dried by fusion, gave on combustion 0.1172 gm. of H_2O and 0.2774 gm. of CO_2.

1.0542 gm. of substance, dried by fusion, required for neutralization 7.98 cc of 0.5 N NaOH corresponding to a molecular weight of 266.

No. 133.   0.1010 gm. of substance, dried by fusion, gave on combustion 0.1170 gm. of H_2O and 0.2764 gm. of CO_2.

1.0169 gm. of substance, dried by fusion, required for neutralization 7.97 cc. of 0.5 N NaOH corresponding to a molecular weight of 255.

No. 134.   0.1005 gm. of substance, dried by fusion, gave on combustion 0.1152 gm. of H_2O and 0.2782 gm. of CO_2.

1.0213 gm. of substance, dried by fusion, required for neutralization 7.85 cc. of 0.5 N NaOH corresponding to a molecular weight of 260.

No. 135.   0.1012 gm. of substance, dried by fusion, gave on combustion 0.1194 gm. of H_2O and 0.2786 gm. of CO_2.

1.0058 gm. of substance, dried by fusion, required for neutralization 7.60 cc. of 0.5 N NaOH corresponding to a molecular weight of 264.

No. 136.   0.1008 gm. of substance, dried by fusion, gave on combustion 0.1170 gm. of H_2O and 0.2793 gm. of CO_2.

0.6550 gm. of substance, dried by fusion, required for neutralization, 4.70 cc. of 0.5 N NaOH corresponding to a molecular weight of 278.

The combined low boiling fractions from two distillations were esterified and again distilled. This fraction boiled constantly at 155–159° at a pressure of 1.6 mm. After saponification and recrystallization from acetic anhydride, followed by purification through the sodium salt as described above, an acid corresponding in its analytical data to palmitic acid was obtained.

No. 169.   0.0994 gm. of substance, dried by fusion, gave on combustion 0.1134 gm. of H_2O and 0.2726 gm. of CO_2.

0.9539 gm. of substance, dried by fusion, required for neutralization 7.40 cc. of 0.5 N NaOH corresponding to a molecular weight of 258.

Sample.	Analysis.		Molecular weight.	Melting point.
	H	C		
	per cent	*per cent*		°C.
Calculated for $C_{16}H_{32}O_2$ (palmitic acid)......	12.58	74.92	256	64–65
Found No. 169..........................	12.77	74.79	258	64–65

By esterification and fractional distillation of the higher boiling fractions, an ester was isolated which distilled at 170–175° under a pressure of 1.6 mm. After saponification this substance gave analytical figures, molecular weight, and melting point corresponding to those of stearic acid.

No. 153.   0.1101 gm. of substance, dried by fusion, gave on combustion 0.1248 gm. of H_2O and 0.3062 gm. of CO_2.

0.7749 gm. of substance, dried by fusion, required for neutralization 5.50 cc. of 0.5 N NaOH corresponding to a molecular weight of 282.

Sample.	Analysis.		Molecular weight.	Melting point.
	H	C		
	per cent	*per cent*		°C.
Calculated for $C_{18}H_{36}O_2$ (stearic acid).......	12.76	75.98	284	70–71
Found No. 153..........................	12.69	75.84	282	70.5–71

The Unsaturated Fatty Acid of Lecithin Isolated from This Fraction.

From the acetone mother liquors from which the saturated fatty acids had been removed by filtration, the unsaturated acid was isolated by precipitation as the lead salt. The gummy mass was dissolved in a small volume of ether, and the ethereal solution kept at a temperature of 0°C. over night. Any contaminating saturated salts crystallized under these conditions and were easily removed by filtration. The unsaturated salt was freed from ether, dissolved in toluene, and decomposed with hydrogen sulfide. The liberated acid was freed from acetone-insoluble impurities by repeated solution in acetone followed by filtration. After concentration of this solution, the residual acid was dark brown in color. A Wijs determination showed the following iodine absorption:

No. 72.   0.2863 gm. of substance absorbed 0.25848 gm. of iodine.

	Calculated for $C_{18}H_{34}O_2$.	Found No. 72.
Iodine number	90	90.4

This acid was then hydrogenated by Paal's method. The saturated acid after two recrystallizations from acetone gave an analysis, molecular weight, and melting point corresponding with those of stearic acid as shown by the following figures:

No. 68.   0.1007 gm. of substance, dried by fusion, gave on combustion 0.1162 gm. of H_2O and 0.2812 gm. of CO_2.

0.9476 gm. of substance, dried by fusion, required for neutralization 6.68 cc. of 0.5 N NaOH corresponding to a molecular weight of 284.

Sample.	Analysis.		Molecular weight.	Melting point.
	H	C		
	per cent	*per cent*		°C.
Calculated for $C_{18}H_{36}O_2$ (stearic acid)	12.76	75.98	284	70–71
Found No. 68..........................	12.91	76.15	284	70–71

B. *Fatty Acids from Lecithin Prepared from the Ethereal Extract.*
Fatty Acids of Dihydrolecithin Prepared from This Fraction.

On the acids isolated from a sample of this lecithin, which before hydrolysis had been reduced to the dihydrolecithin, we obtained the

following analytical data, corroborating their identity with those isolated in an analogous manner from the lecithin of egg oil.

No. 156. 0.1022 gm. of substance, dried by fusion, gave on combustion 0.1166 gm. of H_2O and 0.2846 gm. of CO_2.

0.7134 gm. of substance, dried by fusion, required for neutralization 5.20 cc. of 0.5 N NaOH corresponding to a molecular weight of 274.

Sample.	Analysis		Molecular weight.	Melting point.
	H	C		
	per cent	*per cent*		*°C.*
Calculated for $C_{18}H_{36}O_2$ (stearic acid).......	12.76	75 98	284	70–71
Found No. 156..........	12.76	75.88	274	64–65

The Saturated Fatty Acids of Lecithin Cadmium Chloride Prepared from This Fraction.

No difference was apparent between the acids isolated from the cadmium chloride salt of the lecithin of the ethereal extract of egg yolk and those isolated from lecithin of the acetone extract. The mixed esters were fractionated into three fractions and a residue.

Sample.	Boiling point of esters	Analysis of acid.		Molecular weight of acid	Melting point of acid.
		H	C		
	°C.	*per cent*	*per cent*		*°C.*
Mixed esters, No. 140...........		12.20	75.16	267	58–59
First fraction..................	152–170				
Refractionated, No. 144 (No. 159)......................	157–160	13.07	74 95	258	64–65
Residue, No. 145..............		13 21	75 26	262	59–60
Second fraction, No. 146.........	154–175	13.04	74.96		60–61
Residue, No. 157................		13.04	75.93	282	70–70.5

The detailed data on these analyses are as follows:

No. 140. 0.1005 gm. of substance, dried by fusion, gave on combustion 0.1096 gm. of H_2O and 0.2770 gm. of CO_2.

1.0819 gm. of substance, dried by fusion, required for neutralization 8.08 cc. of 0.5 N NaOH corresponding to a molecular weight of 267.

No. 159.   0.0998 gm. of substance, dried by fusion, gave on combustion 0.1166 gm. of H_2O and 0.2743 gm. of CO_2.

0.9948 gm. of substance required for neutralization 7.70 cc. of 0.5 N NaOH corresponding to a molecular weight of 258.

No. 145.   0.1088 gm. of substance, dried by fusion, gave on combustion 0.1190 gm. of H_2O and 0.2782 gm. of CO_2.

1.0080 gm. of substance required for neutralization 7.70 cc. of 0.5 N NaOH corresponding to a molecular weight of 262.

No. 146.   0.1004 gm. of substance, dried by fusion, gave on combustion 0.1170 gm. of H_2O and 0.2760 gm. of CO_2.

No. 157.   0.1007 gm. of substance, dried by fusion, gave on combustion 0.1174 gm. of H_2O and 0.2804 gm. of CO_2.

0.6874 gm. of substance, dried by fusion, required for neutralization 4.87cc. of 0.5 N NaOH corresponding to a molecular weight of 282.

The Unsaturated Acid of Lecithin Prepared from This Fraction.

These results were similar to those obtained on the unsaturated acid of the previous fraction.   The acid was purified by the process described above.   Its iodine value was 87.

. No. 141.   0.3995 gm. of substance absorbed 0.34308 gm. of iodine when titrated according to the method of Wijs.

	Calculated for $C_{18}H_{34}O_2$.	Found No. 141.
Iodine number	90	87

Saturated Fatty Acids from a Sample of Lecithin Prepared from This Fraction.

A sample of the lecithin cadmium chloride used in the preceding experiment was decomposed with ammonium carbonate.   After purification it gave the following analysis:

No. 50.   0.1957 gm. of substance used for a Kjeldahl determination required 2.85 cc. of 0.1 N HCl, equivalent to 0.00399 gm. of N.

0.2936 gm. of substance gave 0.0408 gm. of $Mg_2P_2O_7$.

0.1096 gm. of substance, dried under diminished pressure at temperature of water vapor, gave on combustion 0.1050 gm. of H_2O, 0.2604 gm. of CO_2, and 0.0110 gm. of ash.

	Calculated for $C_{43}H_{80}O_9NP$.	Found No. 50 (calculated ash-free).
	per cent	*per cent*
C...........	65.27	65.56
H............	10.95	10.85
N................................	1.77	2.04
P...........	3.92	3.87

This material was hydrolyzed and the mixed fatty acids after purification through the lead salt analyzed as follows:

No. 173. 0.0995 gm. of substance, dried by fusion, gave on combustion 0.1150 gm. of H_2O and 0.2758 gm. of CO_2.

1.1333 gm. of substance required for neutralization 8.60 cc. of 0.5 N NaOH corresponding to a molecular weight of 264.

Sample No.	Analysis.		Molecular weight.	Melting point.
	H	C		
	per cent	*per cent*		°C.
173	12 93	75.59	264	57–58

ION SERIES AND THE PHYSICAL PROPERTIES OF PROTEINS.

III. The Action of Salts in Low Concentration.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

I. The Difference in the Effect of Acids, Alkalies, and Salts on Proteins.

The data concerning electrolytic dissociation and the behavior of electrolytes in general suggest that it is well to discriminate between solutions of electrolytes in low and in high concentrations. While no sharp line of demarcation can be drawn it will suffice for the problem we are discussing to designate a concentration of electrolytes below $M/16$ as low and those near the solubility limit of one of the components as high. In this paper we intend to deal with solutions of low concentrations; *i.e.*, $M/16$ or less.

It has been noticed by a number of authors that the influence of neutral salts on the physical properties of proteins differs from that of acids and bases; and various attempts have been made to find an expression for this difference. Pauli[1] states that while acids and alkalies form salts with proteins, neutral salts form "adsorption compounds" with "electrically neutral," *i.e.* non-ionized, protein molecules, both ions of the salt being simultaneously adsorbed by the protein molecule. This idea is no longer tenable for salt solutions of low concentration since the writer has shown through his experiments with powdered gelatin that only one (or practically only one) of the two ions of a neutral salt can combine at one time with a protein. At the isoelectric point, *i.e.* at pH 4.7, gelatin can combine with neither ion of a neutral salt; at a pH > 4.7 only the metal ion of the neutral salt can combine with the gelatin, forming metal gelatinate;

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[1] Pauli, W., *Fortschr. naturwiss. Forschung*, 1912, iv, 223.

at a pH < 4.7 only the anion of the neutral salt is capable of combining with the protein, forming gelatin-acid salts.[2]

Lillie has made the statement that while acids and alkalies increase, salts depress the osmotic pressure of gelatin.[3] This statement, while it was the expression of facts actually observed by Lillie, is no longer tenable owing to the fact that the influence of the hydrogen ion concentration of the gelatin solution was not taken into consideration. If we add acid to a gelatin-acid solution of a pH of 3.0 or below, the effect is practically the same as when we add a neutral salt, namely a diminution of the osmotic pressure of the solution; and when we add alkali, e.g. KOH, to a solution of a metal gelatinate of pH 11.0 or above, the effect is also a similar depression of the osmotic pressure as that caused by the addition of KCl. We also get a depression when we add some acid to a solution of metal gelatinate or when we add some alkali to gelatin-acid salts; since in both cases the gelatin is brought nearer to the isoelectric point.

It is also incorrect to speak of an antagonism between the effects of acids and salts, since the facts mentioned show that there is also an antagonism between little and much acid; thus if the pH of a gelatin-acid salt is 3.0 a further addition of acid depresses the osmotic pressure or viscosity. The question then arises, what is the correct expression of the facts in the case?

An analogy with another field of phenomena may be of service. The writer has recently published a series of articles on the influence of electrolytes on the rate of diffusion of water through collodion membranes, which have shown that water diffuses through such a membrane as if the particles of water were positively charged. When pure water is separated from a solution of an electrolyte of not too high a concentration, the positively charged particles of water diffuse through the collodion membrane into the solution as if they were attracted by the anion and repelled by the cation with a force increasing with the valency of the ion. In this case the oppositely charged ions of an electrolyte influence the rate of diffusion of water through the membrane in an opposite sense.

[2] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 237; *Science*, 1920, lii, 449.

[3] Lillie, R. S., *Am. J. Physiol.*, 1907–08, xx, 127.

The second fact which was brought out was that the relative strength of the apparent attractive and repelling action of the oppositely charged ions of an electrolyte upon the electrically charged particles of water varies with the concentration of the electrolyte in the solution. In the lower concentrations of the electrolyte the attractive action of the anion upon the positively charged particles of water increases more rapidly with increasing concentration of the salt than the repelling action of the cation; while when a certain concentration is reached the repelling action of the cation upon the positively charged particles of water increases more rapidly with a further increase in the concentration of the salt than the action of the anion.[4]

These facts offer an analogy which is helpful in understanding the difference in the action of acids and alkalies on the one hand, and of neutral salts on the other upon the physical properties of proteins.

When acids or alkalies are added to isoelectric gelatin both ions of the acid or alkali influence the physical properties of proteins but in an opposite direction. When we add acid to isoelectric protein the hydrogen ions increase, the anions depress the osmotic pressure and viscosity of the protein solution (and this depressing action increases with the valency of the anion of the acid). As long as little acid is added to isoelectric protein the augmenting action of the hydrogen ion on these properties increases more rapidly with increasing concentration of the acid than the depressing action of the anion; while when the pH of the solution falls below 3.3 or 3.0 the reverse is the case. This causes the drop in the curves for osmotic pressure, viscosity, and swelling below a pH of 3.0.

When we add alkali to isoelectric protein the OH ions (or the diminution of the concentration of hydrogen ions) increase the osmotic pressure, viscosity, etc., of the solution of metal proteinate while the cation of the alkali depresses these properties with a force increasing with the valency of the cation. In the lowest concentrations of the alkali added the augmenting action of the OH ion on the physical properties of the metal proteinate increases more rapidly with the concentration than the depressing effects of the cation of the alkali; while in higher concentrations, *i.e.* as soon as the pH becomes about 10.0 or 11.0, the reverse is the case.[5]

[4] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 173.
[5] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 85, 247.

When, however, neutral salts are added to protein solutions we no longer notice an opposite effect of the oppositely charged ions. When neutral salts are added to isoelectric gelatin no effect is noticed as long as the concentration of salt does not reach the value required for precipitation. When neutral salt is added to a protein solution on either side of its isoelectric point only a depressing action of that ion which has the opposite sign of charge as the protein ion is observed. No augmenting action of the ion with the same sign of charge as the protein is noticeable. Thus if we add $CaCl_2$ or Na_2SO_4 to a solution of gelatin chloride or gelatin nitrate we observe only a depressing effect of the Cl or SO_4 ion but no augmenting effect of the Ca or Na ion; while when we add these salts to a solution of a metal gelatinate we observe only a depressing effect of the Ca or Na ion but no augmenting effect of the anion. We shall first show that this is the correct expression for the difference in the action of acids and bases on the viscosity of proteins on the one hand and of neutral salts on the other.

A 2 per cent solution of isoelectric gelatin is prepared and brought to a pH of 4.0. The solution is made 1 per cent in regard to the originally isoelectric gelatin by adding to 50 cc. of the 2 per cent solution either 50 cc. of H_2O or of a salt solution, e.g. NaCl, of different molecular concentration, from M/8,192 to 1 M, taking care that the hydrogen ion concentration remains the same. We determine the viscosity (i.e. the time of outflow through a viscometer) in the way described in a preceding publication and plot the reciprocal of time of outflow (counting that of water as 1) as ordinates over the pH as abscissæ (lower curve, Fig. 1). For the sake of briefness we beg leave to designate this value as specific viscosity.[5] The addition of the NaCl causes only a drop, and no rise in the curve.

If, however, we mix the 2 per cent gelatin solution of pH 4.0 with various concentrations of HCl (upper curve, Fig. 1) instead of with NaCl we do not notice a drop but at first a rise followed by a drop when the concentration of the Cl ion is a little above N/1,000. In Fig. 1 the drop appears at a concentration of about N/256 HCl, but the reader must remember that on account of the fact that part of the acid combined with the gelatin the pH of the solution was about 3.0. In other words, while the addition of H ions increases the vis-

cosity of a solution of gelatin chloride of pH 4.0, the addition of Na ion does not have such an effect, but the Cl ion depresses the viscosity in both cases, no matter whether NaCl or HCl is added to the gelatin
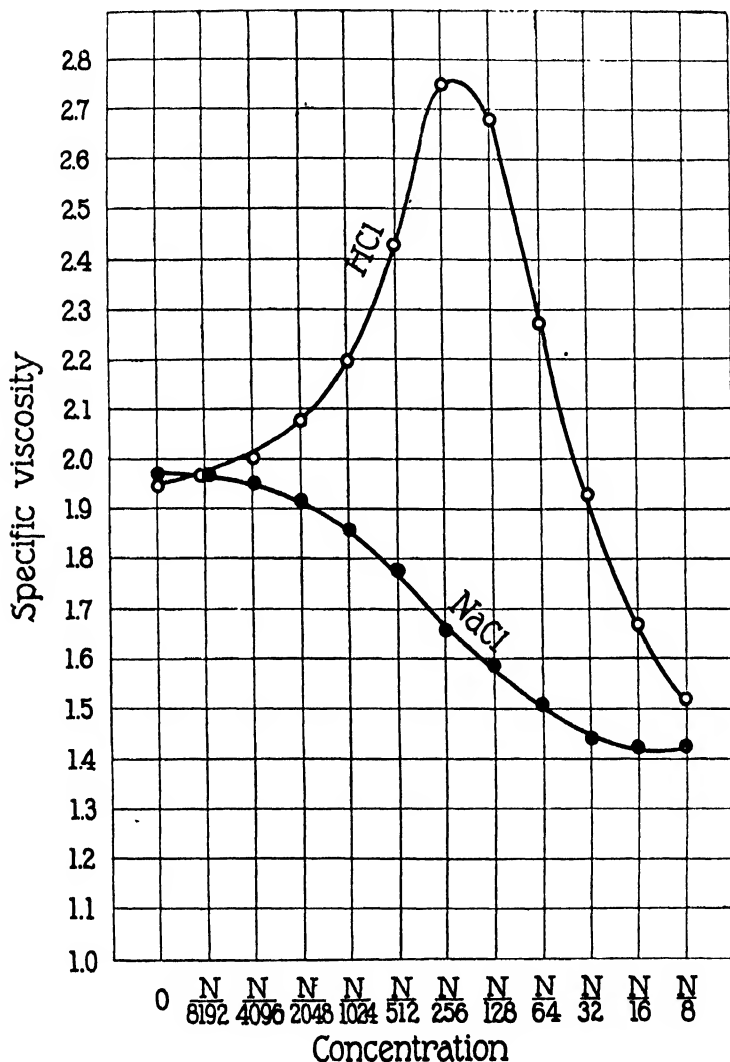
FIG. 1. Difference in the effect of different concentrations of NaCl and of HCl on the specific viscosity of a 1 per cent solution of gelatin chloride of pH 4.0. In the case of NaCl we observe only the depressing effect of the Cl ion; in the case of HCl we notice an augmenting effect of the H ion and a depressing effect of the Cl ion, the latter prevailing as soon as the concentration of acid added is > N/256.

solution; and the depressing action of the Cl ion increases with its concentration.   Moreover, the increase of the viscosity by the H ions stops as soon as the pH of the solution reaches about 3.0.

When we repeat the same experiment with a gelatin solution of pH 3.0, the addition of NaCl immediately causes a drop also (Fig. 2)
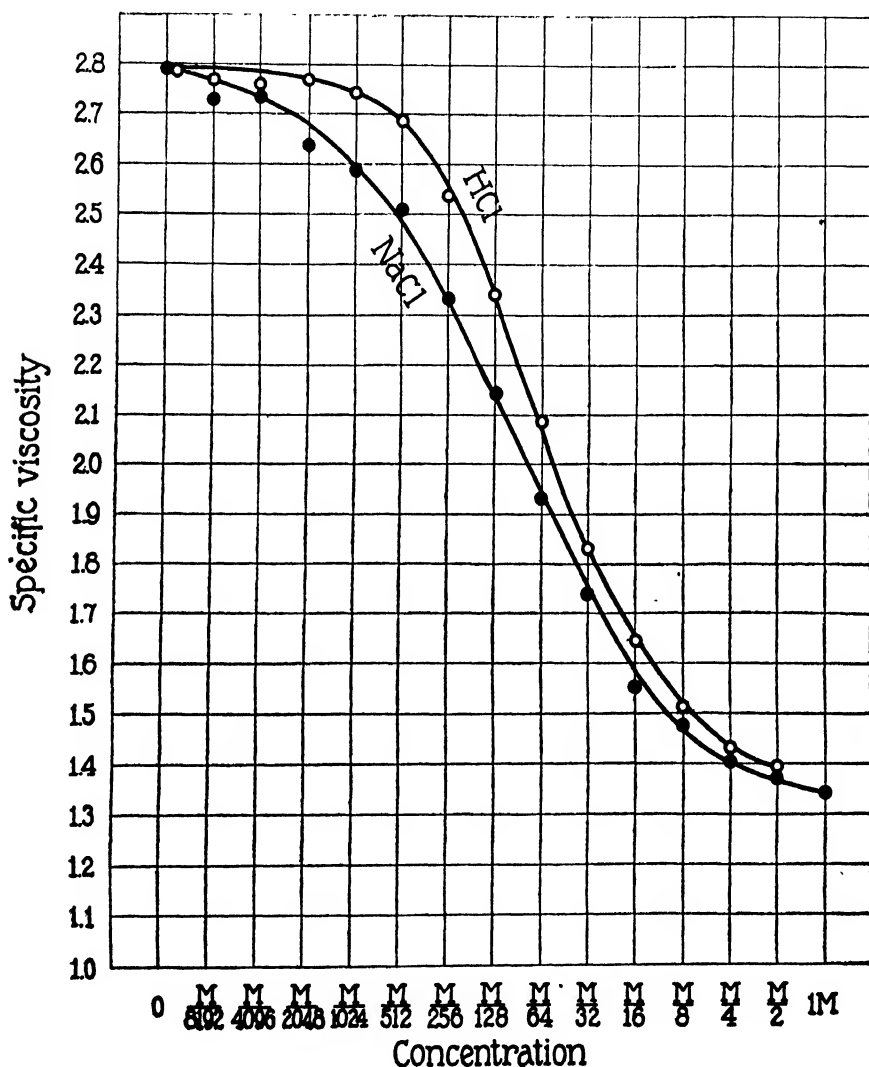
FIG. 2. The specific viscosity of 1 per cent solution of gelatin chloride of pH 3.0 is depressed almost equally by the Cl ion of HCl as of NaCl.   The augmenting effect of the H ion in the case of HCl is no longer noticeable.

while the addition of HCl no longer causes a rise but the drop commences a little later than in the case of NaCl.

When, however, we make the same experiment with a gelatin solution of pH 2.5 (Fig. 3), we notice an immediate drop upon the
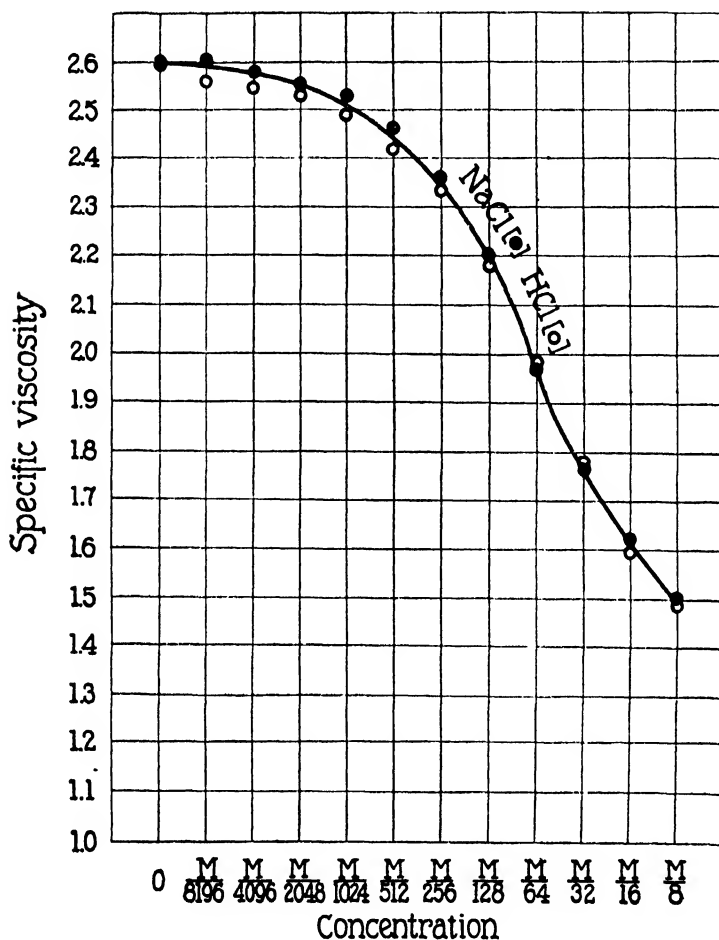
FIG. 3. When the gelatin solution has a pH of 2.5, HCl and NaCl depress the specific viscosity of the gelatin solution to the same degree.

addition of HCl as well as in the case of the addition of NaCl, and the curve for HCl coincides practically with that for NaCl, as our theory demands.

That the depression of the viscosity of gelatin chloride due to the presence of a salt is exclusively determined by the anion of the salt

and that the cation has no augmenting effect is shown in Fig. 4, where the influence of NaCl, CaCl₂, and LaCl₃ upon the viscosity of gelatin of pH 3.0 is represented.   50 cc. of a 2 per cent solution of gelatin chloride of pH 3.0 were added to 50 cc.of a solution of different concentrations of each salt as described, the pH being kept at 3.0.   It is
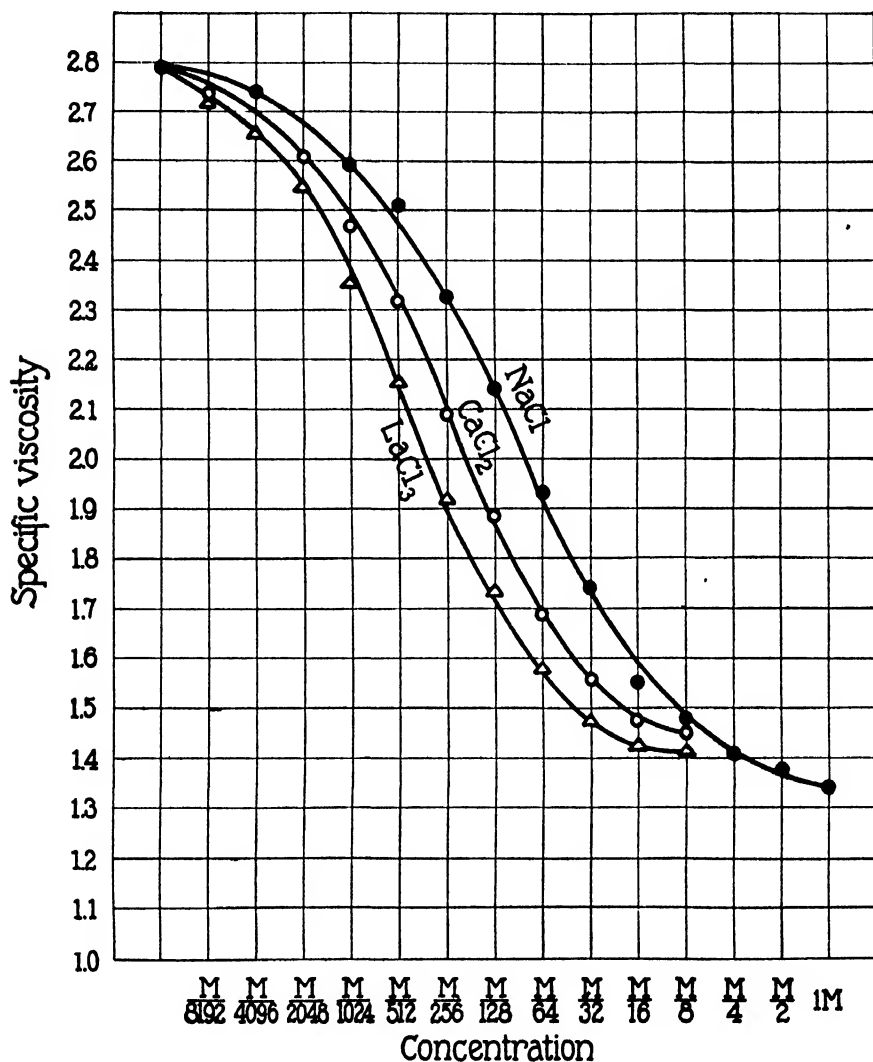
Fig. 4. The depressing effect of equal molecular concentrations of NaCl, CaCl₂, and LaCl₃ on the specific viscosity of 1 per cent gelatin chloride solution of pH 3.0 is in exact proportion to the concentration of the Cl ions in the solution; *i.e.*, as 1:2:3.

obvious from Fig. 4 that the molecular concentrations of NaCl, CaCl$_2$, and LaCl$_3$, which depress the viscosity to the same level are approximately in the ratio of 3:2:1. Thus when the effect of NaCl and CaCl$_2$ is plotted over the same concentration of the Cl ions the curves for the salts become identical (Fig. 5), and the same would be practically
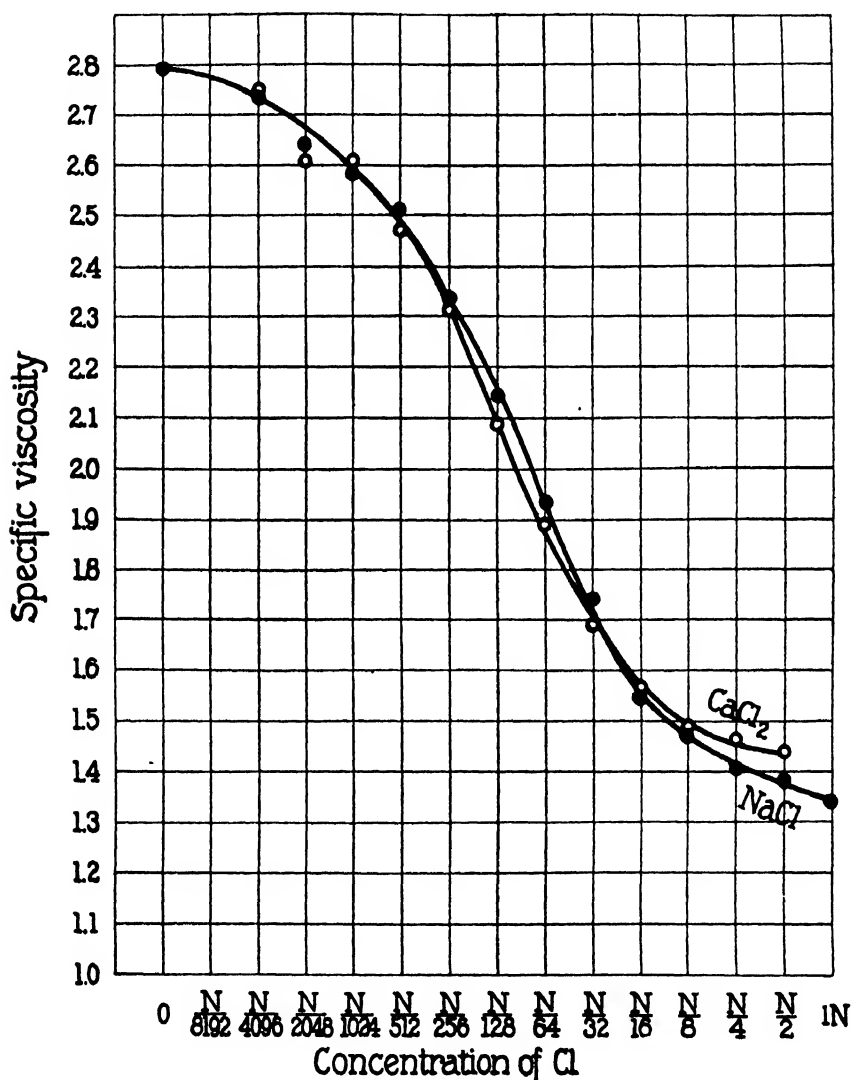
FIG. 5. Showing that NaCl and CaCl$_2$ have the same depressing effect on the viscosity of gelatin chloride of pH = 3.0 when the concentration of Cl ions is the same.

true for the LaCl$_3$ curve. From this it follows that the depressing effect of these three salts on gelatin chloride is practically exclusively a function of the concentration of the Cl ion, while no effect of the cation seems to be noticeable.   In all these cases the pH of the gelatin solution was not altered by the addition of the salt.

When we prepare 1 per cent solutions of gelatin chloride of pH 3.0 in solutions of Na salts with the anion of a weaker acid, e.g. Na$_2$ oxalate, Na$_4$Fe(CN)$_6$, the pH is increased and we are in danger of attributing erroneously a depressing effect to the anion which in reality is caused by the increase in pH.   In Fig. 6 the effects of the addition of equal concentrations of NaCl, Na$_2$SO$_4$, and Na$_4$Fe(CN)$_6$ on gelatin chloride of pH = 3.0 are plotted.   In the case of Na$_4$Fe(CN)$_6$ only the lowest concentrations, from M/8,192 to M/1,024, could be used, since in these only did the pH of the protein solution remain = 3.0. Fig. 6 shows that the depressing effect of these salts increases rapidly with the valency of the anion.   When the concentration of the salt was only M/1,024 a drop in the viscosity was already noticeable. This drop was small in the case of NaCl (from 2.8 to 2.6), was greater in the case of Na$_2$SO$_4$ (from 2.8 to 2.35), and considerably greater in the case of Na$_4$Fe(CN)$_6$ (from 2.8 to 1.5).   The objection might be raised that since Na$_2$SO$_4$ has twice as many cations as NaCl of the same concentration and Na$_4$Fe(CN)$_6$ has four times as many cations, it was the difference in the concentration of the cations which caused the difference in the drop.   This is refuted by the fact that Na$_2$SO$_4$ causes a drop to 1.8 at a concentration of M/256 while NaCl causes the same drop at a concentration of above M/64 which is about four times as high.   If the concentration of the cation were responsible for the drop the two concentrations should be as 1:2.   Na$_4$Fe(CN)$_6$ causes the same drop of the viscosity to 1.8 at a concentration less than M/1,024. Hence the concentration of Na$_4$Fe(CN)$_6$ required to cause the same diminution of the specific viscosity as that caused by M/64 NaCl is less than $\frac{1}{16}$ of the latter, while it should be at the least only $\frac{1}{4}$ if the cation were responsible for the drop.   The depressing effect of the anion seems to increase almost in proportion to the square of its valency, as the Hardy-Whetham rule demands.

We have selected viscosity experiments, but experiments on osmotic pressure and on swelling lead to the same formulation of the difference in the effect of acids and salts.

What has been shown for the effect of acids on the physical properties of proteins can also be shown for the influence of alkalies. Thus the addition of KOH to Na gelatinate of pH 12.0 depressed the
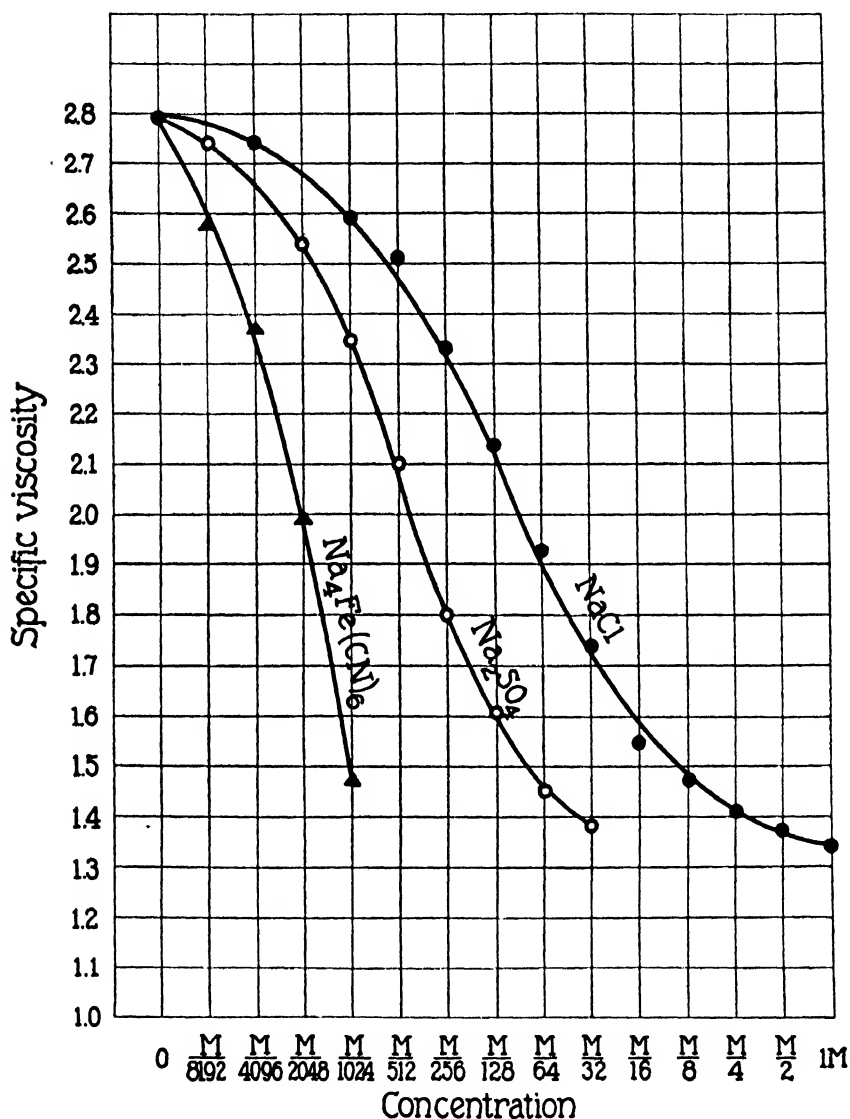
FIG. 6. The relative depressing effect of equal molecular concentrations of NaCl, Na₂SO₄, and Na₄Fe(CN)₆ on the specific viscosity of a gelatin chloride solution of pH 3.0 is approximately as 1:4:16.

viscosity in the same way as the addition of KCl (Fig. 7); while the addition of KOH to Na gelatinate of pH 4.8 to 8.0 increases the viscosity and the addition of KCl to Na gelatinate always depresses the viscosity. The depressing effect of salts on metal gelatinate is due to the cation of the electrolyte added, that of bivalent cations being greater than that of monovalent cations, while the valency of the anion has no effect.

FIG. 7. The depressing effect of KOH and KCl on Na gelatinate of pH 12.0 is practically the same.

The addition of neutral salt to isoelectric gelatin leaves the viscosity and osmotic pressure of the solution practically unchanged.

The cause of this difference in the action of acids, alkalies, and salts is easy to understand on the basis of the purely chemical theory of the behavior of proteins. When we add acid to isoelectric gelatin we transform part of the latter into gelatin-acid salt and the proportion of gelatin-acid salt formed increases with the amount of the acid added. Near the isoelectric point the amount of gelatin-acid salt formed increases very rapidly with the addition of acid, but when

the pH approaches 3.0 the addition of the same amount of acid which near the isoelectric point caused a considerable change now causes only a slight change, while when the pH falls below 3.0 the depressing influence of the anion continues to increase with increasing concentration of the electrolyte.

II. Ion Series and the Action of Salts on Proteins.

We have shown in preceding papers that, as long as the concentration of the electrolyte is not too high, only the sign of the charge and the valency of an ion influence such physical properties as swelling, viscosity, solubility in alcohol, and osmotic pressure of proteins; while all the different ions of the same sign of charge and valency have either the same effect or if there is a difference in effect it is too small to be noticed with our present methods of work.[5] This proof was furnished for the action of acids and alkalies on the physical properties of proteins just mentioned. We now wish to make the proof complete by considering also the action of salts. To do this we are compelled to compare the relative depressing action of low but equal concentrations of different salts upon the physical properties of a gelatin salt, for example gelatin chloride of a definite pH; e.g., 3.0. As can be easily surmised the addition of a salt will in many cases alter the pH of the solution and this alteration will be larger in the case of certain salts, e.g. Na acetate, than in the case of others, e.g. NaCl. Unless we take into consideration these variations in the pH caused by the addition of salts we shall be in danger of erroneously ascribing the influence of a variation in the hydrogen ion concentration to an influence of the nature of the anion. The Hofmeister ion series are due to this error.

The method of our experiments was as follows. 50 cc. of a 2 per cent solution of originally isoelectric gelatin were brought to a pH of 3.0 by the addition of HCl. To this were added 50 cc. of H_2O or of a salt solution of different molecular concentration, and the viscosity of this mixture was measured using those precautions which were described in a preceding paper.

Fig. 8 gives the curves representing the depression of the specific viscosity of a gelatin chloride solution of pH 3.0 by different concentrations of salts with monovalent anion; namely, NaCl, NaH_2PO_4,
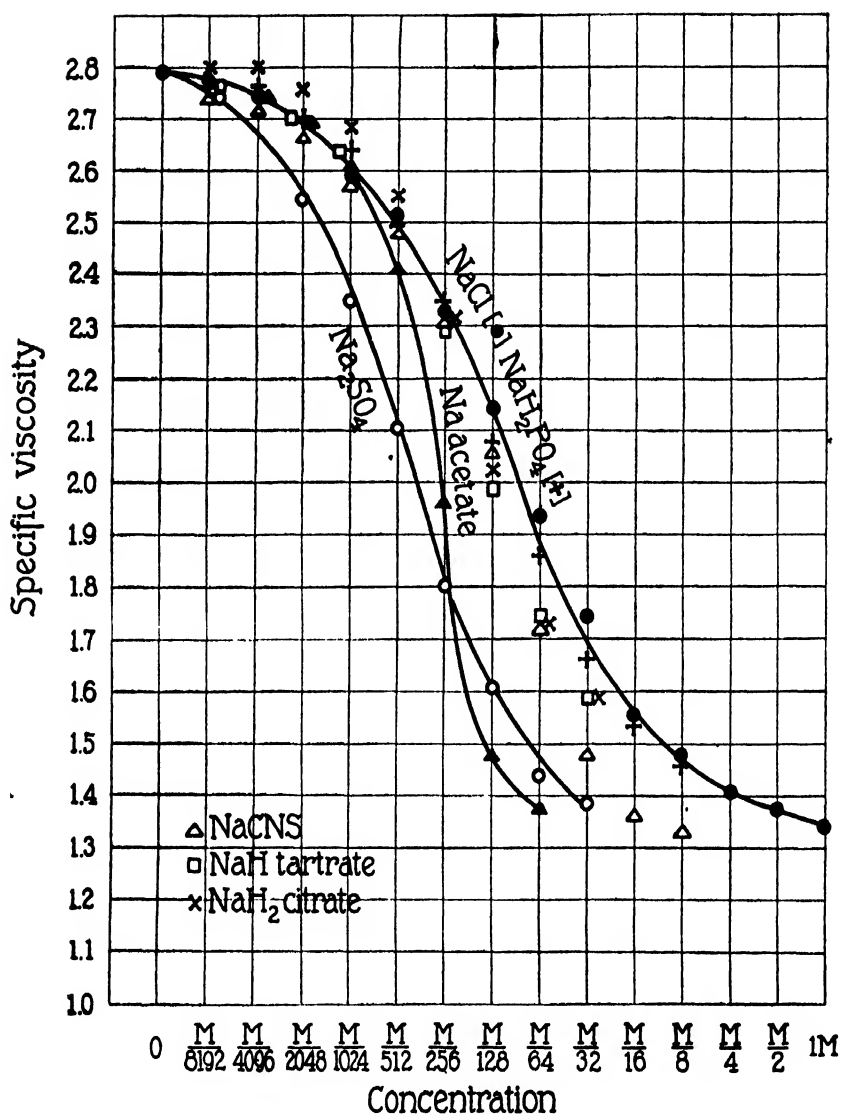
FIG. 8. The depressing effect of different salts with monovalent anion (NaCl, NaH₂PO₄, NaCNS, NaH tartrate, and NaH₂ citrate) on the specific viscosity of 1 per cent solution of gelatin chloride of pH 3.0. The effects of NaCl and NaH₂PO₄ are identical since the pH is not altered by the addition of these salts. The depression in the values for the specific viscosity is greater in the case of Na acetate than in the case of NaCl for the reason that the Na acetate raises the pH of the gelatin solution.

NaCNS, NaH tartrate, NaH₂ citrate, and Na acetate. The curve for Na₂SO₄ is added for comparison. The monosodium salts of weak dibasic and tribasic acids dissociate electrolytically into a Na ion and a monovalent anion, H_2PO_4, H tartrate, H_2 citrate, etc. All the salts mentioned in Fig. 8 are therefore salts with monovalent anion with the exception of Na_2SO_4. Our valency rule demands that the relative depressing effect of these salts (with the exception of Na_2SO_4) should be the same and that deviations from this rule should find their explanation in corresponding deviations of the pH due to the influence of certain of the salts. We will first consider this latter influence as given in Table I, which shows the results of the measurements of pH

TABLE I.

Changes in pH of 1 Per Cent Gelatin Chloride of pH = 3.0 upon Addition of Various Concentrations of Salts.

					Molecular concentrations of salts used.								
	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4
NaCl	3 0	3 0	3 0	3 0	3 0	3 0	3 0	3 0	3 0	3 0	3 0	3.0	3.0
Na₂SO₄	3 0	3.0	3 0	3 0	3 0	3 0	3.0	3.0	3 05	3 1	3.2	3 3	3 35
NaH₂PO₄	3.0	3.0	3 0	3 0	3 0	3 0	3 0	3.1	3 2	3 3	3.4	3 45	3.5
NaCNS	3 0	3 0	3 0	3 0	3.0	3 0	3 1	3.2	3.3	3.6	3 9	4.2	4.4
NaH tartrate	3.0	3.0	3 0	3 0	3 0	3 0	3.1	3 3	3.45	3.5	3 55		
NaH₂ citrate	3 0	3.0	3.0	3 0	3 0	3 1	3 2	3.4	3 6	3 7	3.75		
Na acetate	3.0	3.0	3.0	3.05	3.1	3 3	3.7	4.3	4.6				

in these different gelatin solutions after the addition of salts. The original gelatin chloride solution had a pH of about 3.0 and this was not altered by the addition of NaCl and only slightly by the addition of NaH_2PO_4 in concentrations below M/16. According to the valency rule the curves for the depressing effect of NaCl and NaH_2PO_4 should be almost identical and Fig. 8 shows that this is the case.

Table I shows that NaCNS, monosodium tartrate, and monosodium citrate raise the pH of the solution as soon as the concentration reaches M/128 or more. If we consider this effect, we must expect to find that the drop in the curves for NaCNS, monosodium citrate, and monosodium tartrate is a little steeper in concentrations of M/128 and

above than the curve for the depressing effect of NaCl. Fig. 8 shows that the curves for the depressing effect of these three salts are slightly lower than the curve for NaCl or NaH_2PO_4. The greatest apparent deviation from the valency rule occurs in the curve for Na acetate whose depressing effect is of the order of that of Na_2SO_4.

In the colloidal literature it is always stated that Na acetate acts like Na_2SO_4 and this is interpreted to mean that the acetate anion acts like the bivalent SO_4 anion and not like the monovalent Cl or NO_3 anion.   Table I shows that Na acetate also depresses the hydrogen ion concentration more than NaCl or NaH_2PO_4; $M/64$ Na acetate brings the gelatin solution practically to the isoelectric point, and at the isoelectric point the viscosity of gelatin solution is a minimum. This lowering of the hydrogen ion concentration (and not the alleged influence of the acetate anion) explains the excessive depressing effect of Na acetate.   That this interpretation is correct can be proved in the following way.   We prepare 1 per cent solutions of gelatin acetate of pH 3.3 and gelatin chloride also of pH 3.3.   The specific viscosity of these two solutions was practically the same (both were 1 per cent solutions in regard to originally isoelectric gelatin).   The solution of gelatin acetate of pH 3.3 was made up in various concentrations of Na acetate of pH 3.3.   The Na acetate solution of pH 3.3 was obtained by dissolving $M/16$ Na acetate in $1\frac{1}{2}$ M acetic acid and the various degrees of dilution of this $M/16$ Na acetate solution of pH 3.3 were brought about by dilution with pure acetic acid of pH 3.3.   The non-dissociated molecules of acetic acid have no more depressing influence on the physical properties of proteins than have the molecules of any non-electrolyte.   Fig. 9 gives the curve representing the depressing effect of Na acetate on gelatin acetate of pH 3.3 when the pH is kept constant.

The gelatin chloride solution of pH 3.3 was made up in different concentrations of NaCl and the depressing effect of NaCl on the viscosity of gelatin chloride is also plotted in Fig. 9.   It is obvious from Fig. 9 that the depressing effect of Na acetate and NaCl are identical when the pH is kept constant and identical in both cases.

The same fact was confirmed in a somewhat different way.   A 2 per cent solution of gelatin chloride of pH 3.0 was made up in various concentrations of Na acetate also of pH 3.0.   In order to prepare Na

acetate solutions of pH 3.0 M/4 Na acetate was dissolved in M/4 HCl and the various dilutions required for the experiment were obtained by diluting the mixture of equal parts of M/4 HCl and M/4 Na acetate with M/1,000 HCl.
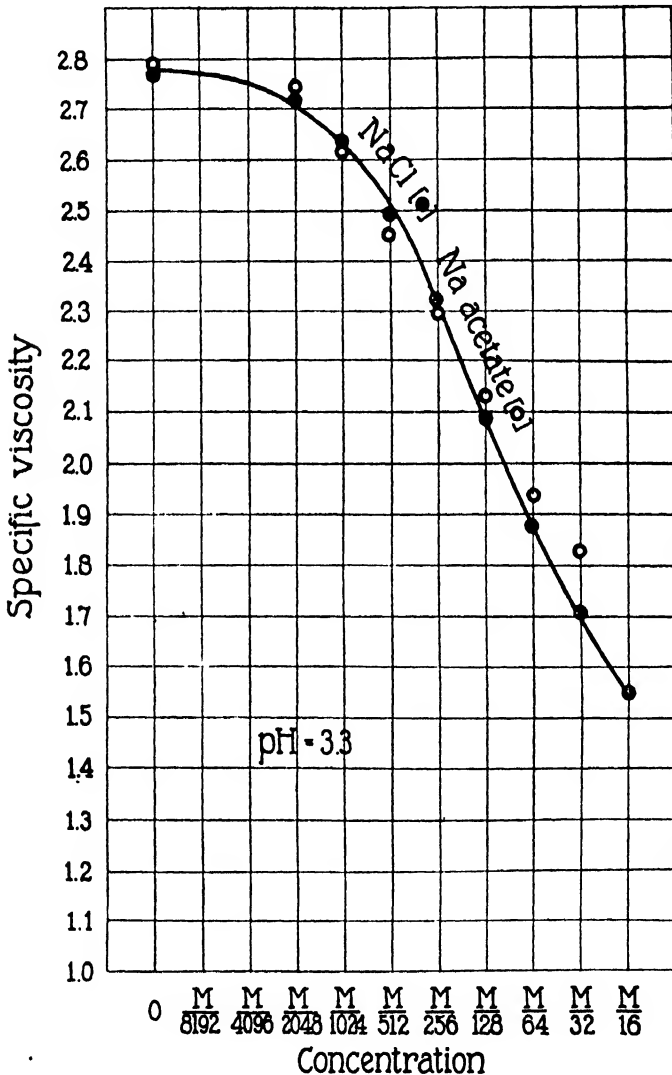
FIG. 9. When the pH is kept equal the depressing effect of equal concentrations of NaCl and Na acetate on the specific viscosity of a 1 per cent gelatin chloride or gelatin acetate solution of pH 3.3 is the same.

The 2 per cent gelatin chloride solution of pH 3.0 was diluted with 50 cc. of this mixture so that the resulting 1 per cent gelatin chloride solution of pH 3.0 contained various concentrations of Na acetate (or
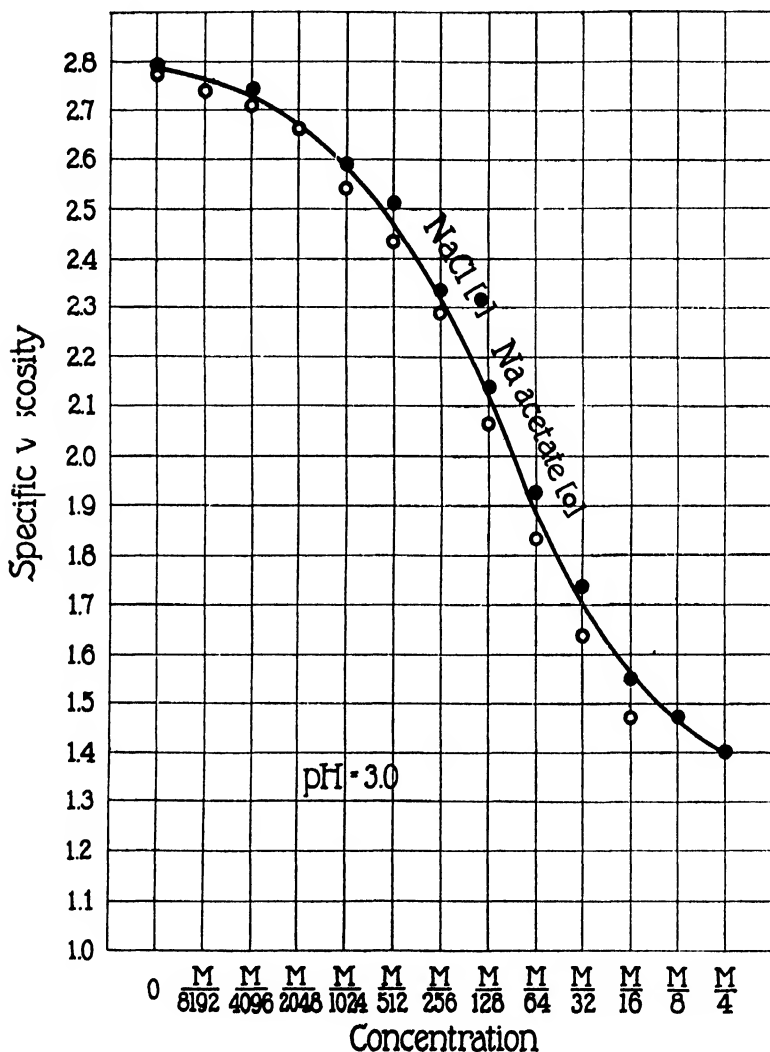
FIG. 10. See legend of Fig. 9, except that the pH of gelatin solution is 3.0.

more correctly of NaCl and Na acetate).   The curve representing the depressing effect of this salt is given in Fig. 10, and is shown to be identical with the curve representing the depressing effect of the addition of NaCl to gelatin chloride of pH 3.0.

We can, therefore, state that sodium acetate has the same effect on the viscosity of gelatin chloride as the addition of any other salt with monovalent anion, and that the anomalous effect ascribed to the acetate anion in the colloidal literature is in reality due to the depression of the hydrogen ion concentration of the gelatin solution by the Na
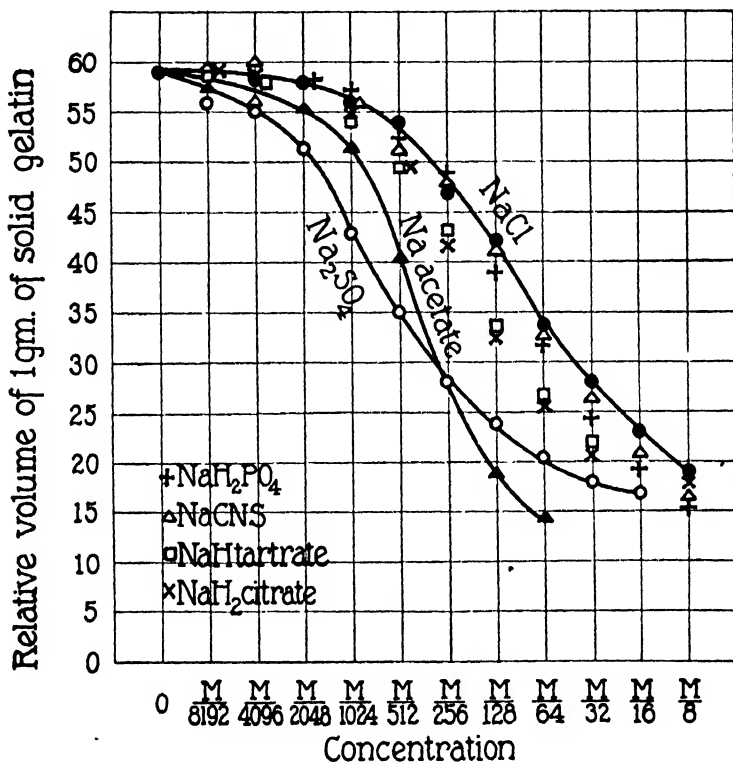
Fig. 11. Showing that the depressing effect of salts with monovalent anion on the swelling of gelatin chloride of pH 3.3 is similar to that on the specific viscosity. All salts with monovalent anion depress the swelling of gelatin chloride to the same extent, the seeming deviation from this rule being due to variation in the pH of the gelatin solution caused by buffer salts.

acetate which is a buffer salt. The failure to recognize the buffer character of salts, like the acetates, citrates, and tartrates, has led to the error of the Hofmeister ion series. In reality we find our valency rule confirmed whereby all salts with an anion of the same valency have the same relative depressing effect on the viscosity of a gelatin chloride solution if the pH of the solution is kept constant.

What has been demonstrated for the effect of these salts on the viscosity of gelatin solutions holds also for their effect on the swelling of gelatin. The same volumetric method for measuring the swelling effect was used which was described in the preceding paper. Fig. 11 gives the relative depressing effect of NaCl, NaH_2PO_4, NaCNS, monosodium tartrate, monosodium citrate, and Na acetate on the swelling of gelatin chloride of pH 3.3 (the curve for Na_2SO_4 is added for comparison), and Table II gives the variation of the pH of the gelatin caused by the addition of these salts. Our theory demands that all these salts (except Na_2SO_4) should depress the swelling of gelatin chloride of pH 3.3 to the same amount, and that deviations from this

TABLE II.

Changes in pH of 1 Per Cent Gelatin Chloride of pH = 3.3 upon Addition of Various Concentrations of Salts.

	Molecular concentrations of salts used.											
	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
NaCl..................	3.3	3 3	3 3	3 3	3.3	3.3	3.3	3.3	3 3	3.3	3.3	3.3
Na_2SO_4...............	3.3	3.3	3.3	3.3	3 3	3 3	3.3	3.3	3.35	3.4	3.5	3.6
NaH_2PO_4.............	3 3	3.3	3.3	3.3	3.3	3.3	3 3	3 3	3.4	3.5	3.6	3.7
NaCNS................	3.3	3 3	3.3	3 3	3 3	3 3	3 3	3.3	3.3	3.3	3.35	3.4
NaH tartrate...........	3 3	3.3	3.3	3 3	3.3	3.4	3.5	3.5	3.6	3.7	3.7	3.7
NaH_2 citrate...........	3.3	3.3	3 3	3.3	3.3	3 4	3.5	3.6	3.8	3.85	3.9	3.9
Na acetate.............	3.3	3.3	3 3	3.4	3.45	3.5	3.8	4.3	4.8	5.2	5.4	5.5

rule must find their explanation in variations of pH caused by the addition of salt. Table II shows that the variations in pH are small for NaCl, NaCNS, and NaH_2PO_4 and hence the curves for the depressing effect of these three salts upon the swelling of gelatin are almost identical, as the valency rule demands. Monosodium citrate and tartrate have a greater depressing effect on the hydrogen ion concentration and Na acetate has a still greater depressing effect than these two salts. This explains the apparent deviation of the curves for these three salts from the valency rule.

Salts like disodium tartrate, disodium oxalate, and trisodium citrate offer an opportunity for an interesting test for our theory on account

of the difference between the electrolytic dissociation of weak dibasic or tribasic acids and the salts of the same acids. A weak dibasic
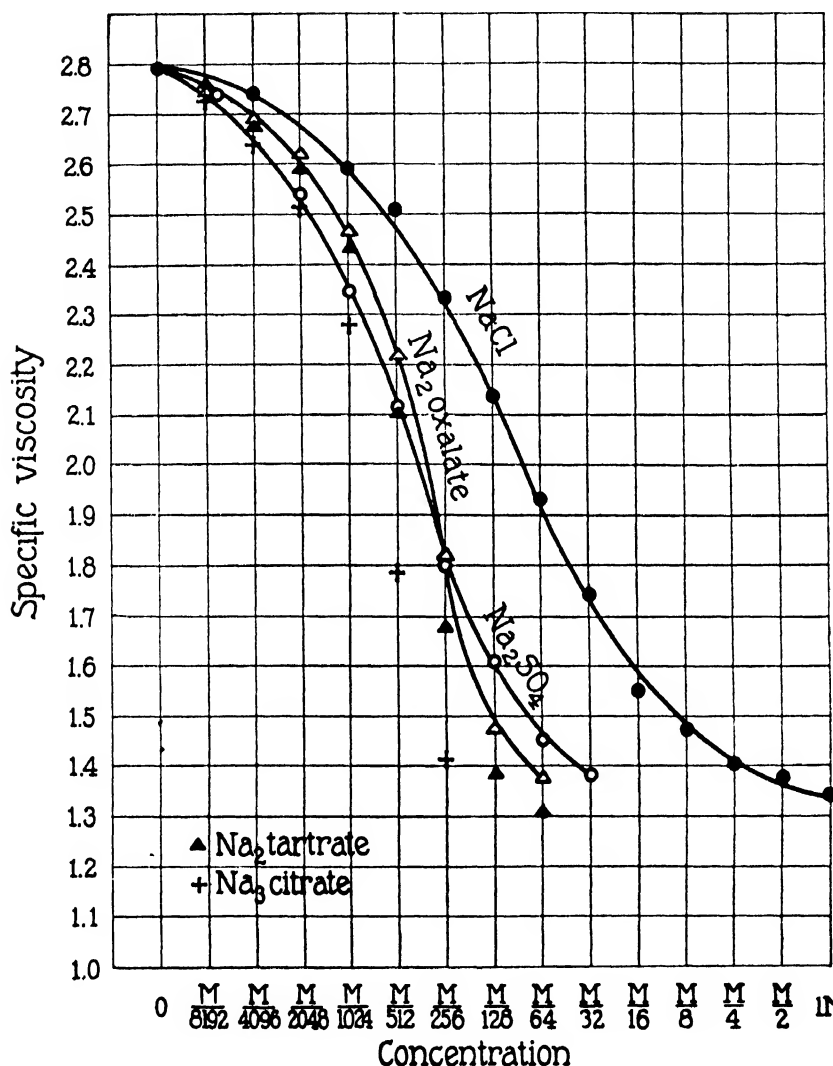
FIG. 12. Bivalent anions have an equally depressing effect as SO₄ on the specific viscosity of a 1 per cent gelatin chloride solution of pH 3.0.

acid like tartaric gives off one hydrogen ion easily but not both; sodium tartrate gives off both Na ions more readily. This is comprehensible on the idea that the oppositely charged ions in a molecule

are held together by electrostatic forces and that these forces are stronger in the case of a hydrogen ion which is free from electrons than in the case of a Na ion where the positive nucleus is separated by two shells of electrons from the valency electron of the oppositely charged ions by which it is held. For this reason the tartaric acid, or the oxalic acid, anion combines with proteins in the form of a monovalent acid tartrate ion while we should expect the anion of disodium tartrate or disodium oxalate to be a bivalent anion. Hence the tartrate and oxalate anions should act like the sulfate anion when disodium tartrate or disodium oxalate are added to a protein solution. This is confirmed, as Fig. 12 shows. The curves for the depressing effect of Na_2 oxalate and Na_2 tartrate practically coincide with the curve for

TABLE III.

Changes in pH of 1 Per Cent Gelatin Chloride of pH = 3.0 upon Addition of Various Concentrations of Salts.

	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4
						Molecular concentrations of salts used.							
NaCl.............	3.0	3.0	3 0	3 0	3 0	3 0	3 0	3 0	3.0	3.0	3 0	3.0	3.0
Na_2SO_4..........	3.0	3 0	3 0	3 0	3.0	3 0	3.0	3.0	3.05	3 1	3.2	3.3	3.35
Na_2 oxalate........	3 0	3.0	3 0	3 05	3 1	3 3	3.5	3 9	4.2	4.4			
Na_2 tartrate.......	3 0	3 0	3 0	3 1	3 2	3.3	3.7	4 0	4.35	4.7	4 8	5 0	
Na_3 citrate........	3.0	3.0	3.05	3.2	3 3	3 8	4.4	5.2	5.7	6.1			

the depressing effect of Na_2SO_4 on the viscosity of gelatin solution, except in concentrations greater than M/256 where disodium tartrate and disodium oxalate cause also a depression of the hydrogen ion concentration (Table III) and where therefore the curves for these two salts drop more rapidly than the curve for Na_2SO_4. Hence disodium oxalate and disodium tartrate act like Na_2SO_4, while, as Fig. 8 shows, monosodium tartrate, monosodium citrate, and monosodium phosphate act like NaCl (if the necessary corrections for pH are made).

The osmotic pressure, viscosity, and swelling of Na gelatinate should be depressed by the cation of a salt and the more so the higher the valency of the cation. Fig. 13 shows that this is true for the swelling of Na gelatinate of pH about 9.3. The molecular concentration in

which the swelling is depressed by the same amount is about half as great for Na_2SO_4 as for NaCl (for molecular concentrations from $M/256$ to $M/32$), proving that the Na ion is responsible for the depression, while it is about eight times as high for NaCl as for $CaCl_2$. The pH of the gelatin was practically the same in all solutions.

All these data confirm our valency rule, whereby ions of the same valency and the same sign of charge have, in the same concentration, the same depressing effect on osmotic pressure, swelling, solubility in
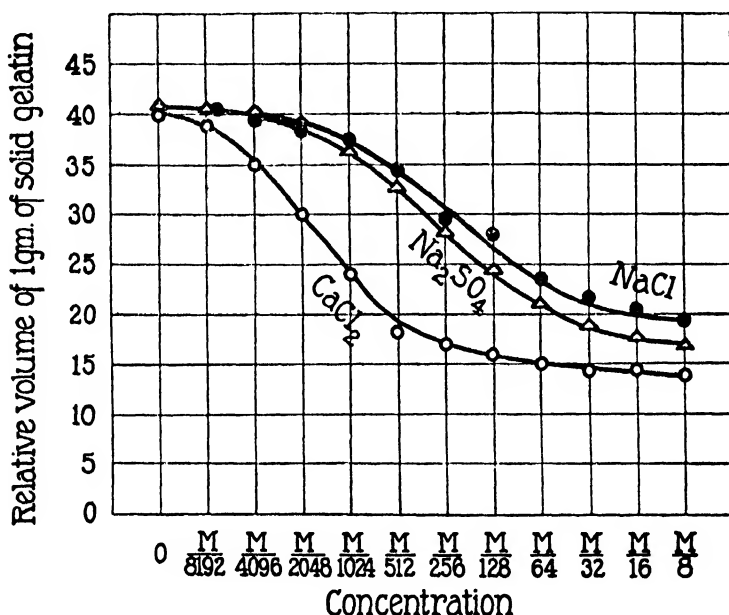
FIG. 13. The depressing effect of neutral salts on the swelling of Na gelatinate of pH about 9.3 is due to the cation of the salt, the depressing effect of NaCl being half as great as that of Na_2SO_4 of equal molecular concentration of Na_2SO_4 while that of $CaCl_2$ is considerably greater owing to the fact that Ca is bivalent.

alcohol, and viscosity of proteins; while the depressing effect increases rapidly with the valency. The Hofmeister ion series are due to the neglect of measuring the influence of the salts on the hydrogen ion concentration of the gelatin solutions. This neglect has given rise to the statement that salts, like sodium acetate, have the same depressing effect on the physical properties of proteins as the sulfates. A number of attempts to apply colloid chemistry to biology, pathology, and medicine are based on such errors.

SUMMARY.

1. Ions with the opposite sign of charge as that of a protein ion diminish the swelling, osmotic pressure, and viscosity of the protein. Ions with the same sign of charge as the protein ion (with the exception of H and OH ions) seem to have no effect on these properties as long as the concentrations of electrolytes used are not too high.

2. The relative depressing effect of different ions on the physical properties of proteins is a function only of the valency and sign of charge of the ion, ions of the same sign of charge and the same valency having practically the same depressing effect on gelatin solutions of the same pH while the depressing effect increases rapidly with an increase in the valency of the ion.

3. The Hofmeister series of ions are the result of an error due to the failure to notice the influence of the addition of a salt upon the hydrogen ion concentration of the protein solution. As a consequence of this failure, effects caused by a variation in the hydrogen ion concentration of the solution were erroneously attributed to differences in the nature of the ions of the salts used.

4. It is not safe to draw conclusions concerning specific effects of ions on the swelling, osmotic pressure, or viscosity of gelatin when the concentration of electrolytes in the solution exceeds $M/16$, since at that concentration the values of these properties are near the minimum characteristic of the isoelectric point.

Note.—The solutions of 1 per cent isoelectric gelatin were prepared by bringing 1 gm. of dry gelatin to the isoelectric point according to the method described in previous papers. It was found that in this process about 20 per cent of the gelatin was lost, so that the originally 1 per cent gelatin solution contained in reality only about 0.8 per cent isoelectric gelatin. This does not affect the contents of the paper or the conclusions since the gelatin concentration used was always the same.

ELECTRIFICATION OF WATER AND OSMOTIC FLOW.*

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I.

The exchange of water and solutes between the cell and the surrounding fluid is one of the important factors in the mechanism of life, and a complete theory of the osmotic flow is therefore a postulate of biology. It was a marked advance when the experiments of Pfeffer and de Vries led van't Hoff to the formulation of the modern theory of osmotic pressure. According to this theory the molecules of the solute behave like the molecules of a gas in the same volume and at the same temperature, and the gas pressure of the solute measures the "attraction" of a watery solution for pure water through a strictly semipermeable membrane. Yet it is obvious to-day that in a liquid the electrical forces between solvent and solute must play a rôle and no adequate provision is made for these forces in van't Hoff's law. Traube rejected van't Hoff's theory altogether, suggesting instead that the osmotic flow was from the liquid with lower to the liquid with higher surface tension (and higher intrinsic pressure).

Tinker has shown that van't Hoff's theory for osmosis holds strictly only in the case of *ideal* solutions, *i.e.*, when the process of solution occurs without heat of dilution and change in volume, but that in the case of *non-ideal* solutions Traube's ideas explain the deviations from the gas law which are bound to occur. When two different *ideal* solutions containing equal numbers of particles of solute in equal volume are separated by a strictly semipermeable membrane, equal numbers of molecules of water will diffuse simultaneously in opposite directions through the membrane and no change in volume will occur. When, however, the same experiment is made with two *non-ideal*

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* Presidential address prepared for the Chicago meeting of the American Society of Naturalists, December 30, 1920.

solutions containing equal numbers of molecules in equal volume, the result is different. As Tinker has demonstrated mathematically, in this case the flow of water must be from the solution having the lower intrinsic pressure and lower surface tension to the solution with higher intrinsic pressure and higher surface tension. This is what Traube claims, and his theory explains therefore, as Tinker points out, the deviations from the gas law in the case of *non-ideal* solutions, but it does not prove that the gas law of osmotic flow does not hold in the case of *ideal* solutions and Traube's theory can not therefore replace van't Hoff's theory.

II.

There is a second group of forces not taken into consideration in van't Hoff's law, namely the influence of the chemical nature of the membrane on the solvent. These forces become noticeable when the membrane separating the solution from the pure solvent is not strictly semipermeable. When water is in contact with a membrane it undergoes as a rule an electrification and this electrification of the particles of water plays a great rôle in the rate of the osmotic flow when the solution into which the water diffuses is an electrolyte. The assumption that water diffusing through a. membrane is as a rule, electrified, is justified by a large number of observations. Quincke demonstrated that when water is pressed through capillary tubes it is found to be electrically charged (the sign of charge being more frequently positive); while the tube has the opposite sign of charge, *e.g.*, negative, when the water is positively charged. When two solutions of weak electrolytes are separated by a membrane (which may be considered as a system of irregular capillary tubes) an electric current causes water to migrate to one of the two poles, according to the sign of its charge. By this method of so-called electrical endosmose it can be shown that water diffuses through collodion membranes in the form of positively charged particles. Collodion bags, cast in the shape of Erlenmeyer flasks, are filled with a weak and neutral solution of an electrolyte, *e.g.*, $M/256$ Na_2SO_4, and dipped into a beaker filled with the same solution of $M/256$ Na_2SO_4. The opening of the collodion bag is closed with a rubber stopper perforated by a glass tube serving as a manometer. When a platinum wire,

forming the negative electrode of a constant current, is put through the glass tube into the collodion bag while the other pole of the battery dips into the outside solution, the liquid in the glass tube rises rapidly with the potential gradient between the two electrodes. The water therefore migrates through the collodion membrane in the form of positively charged particles. The writer has made a number of experiments[1] concerning the osmotic flow through collodion membranes, and it is the purpose of this address to give a brief survey of the results.

<div align="center">III.</div>

When a collodion bag is filled with a solution of a crystalloid, *e.g.*, sugar or salt, and dipped into a beaker containing pure water the pure water will diffuse into the solution and the level of liquid in the capillary glass tube serving as a manometer will rise. At the same time particles of the solute will diffuse out of the bag (except when the solute is a protein solution or a solution of some other colloid). The concentration of a crystalloid solute inside the collodion bag will therefore become constantly smaller until finally the solution is identical on both sides of the membrane. Nevertheless the relative force with which a given solution inside the collodion bag "attracts" the pure water into which the bag is dipped can be measured by the initial rise in the level of water in the manometer, before the concentration of the solution has had time to diminish to any great extent through diffusion. Since in the first minutes accidental irregularities are liable to interfere with the result, we measure the rise in the level of liquid in the manometer during the first 20 minutes.

If the initial rise of level of liquid in the solution is thus measured it is noticed that it occurs approximately in proportion with the concentration of the solution when the solute is a non-electrolyte. The rate of diffusion of pure water into a solution of cane sugar through a collodion membrane is therefore approximately a linear function of the concentration of the solute within the limits of O and 1 M. This is what we should expect on the basis of van't Hoff's theory of osmotic pressure.

[1] Loeb, J., *J. Gen. Physiol.*, 1918–19, I., 717; 1919–20, II., 87, 173, 273, 387, 563, 659, 673.

If, however, a watery solution of an electrolyte is separated from pure water by a collodion membrane, water diffuses into these solutions as if its particles were positively charged, and as if they were attracted by the anion of the electrolyte in solution and repelled by the cation with a force increasing with the valency of the ion (and another property of the ion to be discussed later).

Pure water diffuses into a $M/128$ solution of NaCl through a collodion membrane more rapidly than it diffuses into a $M/64$ solution of cane sugar; water diffuses into a $M/192$ solution of Na_2SO_4 or Na_2 oxalate still more rapidly than into a $M/128$ solution of NaCl; and into a $M/256$ solution of Na_3 citrate, water diffuses more rapidly than into a $M/192$ solution of Na_2SO_4 and, into a $M/320$ solution of $Na_4Fe(CN)_6$ still more rapidly than into a $M/256$ solution of Na_3 citrate. Assuming complete electrolytic dissociation of the electrolytes in these cases, the influence of the five solutions mentioned should be identical according to van't Hoff's theory. We notice, instead, that the "attraction" of the solutions for water increases with the valency of the anion. This is true for all neutral solutions of salts contained in a collodion bag, regardless of the nature of the cation.

If a collodion bag containing a neutral solution of a salt with bivalent cation, e.g., $M/192$ $CaCl_2$ or $MgCl_2$, or with a trivalent cation, e. g., $M/256$ $LaCl_3$, is dipped into a beaker with pure water we notice no rise in the level of water in the manometer. In solutions with bivalent or trivalent cations the repulsion of the cation equals or exceeds therefore the attraction of the anion for the positively charged particles of water diffusing through the pores of the collodion membrane. Hence we conclude from these (and numerous similar) experiments that the particles of water diffuse through a collodion membrane as if they were positively charged and as if they were attracted by the anion of an electrolyte and repelled by the cation with a force increasing with the valency of the ion.

It seemed of interest to find that concentration of a cane sugar solution which just suffices to prevent the diffusion of water into a given solution of an electrolyte. Into each of a series of beakers, all containing the same neutral salt solution, e.g., $M/192$ Na_2SO_4, was dipped a collodion bag containing a cane sugar solution of different concentration, from $M/128$ to 1 M, and it was observed in which of

these sugar solutions the level in the manometer rose during the first 10 minutes, in which it fell, and in which it remained constant. It was found that the cane sugar solution which was just able to balance the "attraction" of the $M/192$ solution of Na_2SO_4 for water had to have a concentration of about or over $M/4$. If the gas pressure effect alone determined the relative attraction of the two solutions for water the concentration of the sugar solutions required to osmotically balance the $M/192$ solution of Na_2SO_4 should have been $M/64$ (or slightly less). Hence the sugar solution balancing osmoti-

TABLE I.

Approximate Concentration of a Solution of Cane Sugar Required to Balance the Osmotic Attraction of the Following Solutions of Electrolytes for Water.

Molecular Concentration.	Electrolyte Used.	Approximate Molecular Concentration of Balancing Solution of Cane Sugar.
$M/128$	KCl	$M/8$
$M/192$	K_2SO_4	Between $M/4$ and $M/2$
$M/192$	K_2 oxalate	$M/2$
$M/192$	K_2 tartrate	Slightly above $M/2$
$M/256$	K_3 citrate	Slightly above $3M/4$
$M/128$	RbCl	$M/4$
$M/128$	KCl	$M/8$
$M/128$	NaCl	$M/8$
$M/128$	LiCl	Slightly above $M/32$
$M/192$	$MgCl_2$	$M/64$
$M/192$	$CaCl_2$	Below $M/64$
$M/192$	$SrCl_2$	Below $M/64$
$M/192$	$BaCl_2$	$M/64$
$M/192$	$CoCl_2$	Below $M/64$
$M/192$	$MnCl_2$	Below $M/64$

cally a $M/192$ Na_2SO_4 solution was found to be 16 times more concentrated than the theory of van't Hoff demands. This high concentration of cane sugar was needed to overcome the powerful "attractive" influence of the anions of a $M/192$ solution of Na_2SO_4 for the positively charged particles of water. Table I shows the results of a few such experiments. The solution of the electrolyte was in these experiments always theoretically isosmotic with a $M/64$ cane sugar solution (on the assumption of complete electrolytic dissociation). The data contained in Table I have only a qualitative value since

no attempt at an exact determination of the concentration of the balancing sugar solutions was made. The data show, however, that the "attraction" of $M/128$ KCl for positively charged particles of water is eight times as great, that of K_2SO_4 sixteen times as great, and that of $M/256$ K_3 citrate almost forty-eight times as great as that of $M/64$ cane sugar; while the "attraction" of a $M/192$ solution of a salt with a bivalent cation and monovalent anion, like $MgCl_2$, for water is not greater than that of a $M/64$ solution of cane sugar.

These experiments then prove that the rate of diffusion of water from the side of pure water through a collodion membrane into a solution of an electrolyte increases with the valency of the anion and diminishes with the valency of the cation. They give also a rough idea of the relative influence of these ions upon the rate of diffusion of positively charged water through the pores of the collodion membrane from the side of pure water to the side of the solution.

A second fact brought out in these experiments was that the relative influence of the oppositely charged ions of an electrolyte in solution upon the rate of diffusion of positively charged water from the side of pure water to the side of the solution is not the same in all concentrations. Beginning with the lowest concentrations, the "attractive" effect of the anion for positively charged water increases more rapidly with increasing concentration than the "repulsive" effect of the cation until the concentration of the electrolyte is about $M/256$; from then on the "repulsion" of the cation upon positively charged water increases more rapidly than the "attractive" effect of the anion. As a consequence we can say that in concentrations of neutral salts between $M/256$ and $M/8$ the "attraction" of the solution for water diminishes with increasing concentration. This is the reverse of what we should expect if the gas law alone determined the attraction of water by solutions of electrolytes. When the concentration of the solution is $M/8$, the apparent electrostatic effects of the ions upon the positively charged particles of water disappear and for concentrations above $M/8$ the curves for the attraction of water by electrolytes and by sugar solutions show less difference.

We have already mentioned the fact that the valency of the ion is not the only quantity which determines its influence on the rate of diffusion of water through a collodion membrane. In addition

to the valency (or the number of electrical charges) a second quantity of the ion enters which may be designated provisionally as the influence of the radius of the ion. In the case of monovalent and monatomic cations the retarding influence on the rate of diffusion of positively charged particles of water through the collodion membrane from the side of pure water into a solution increases inversely with the radius of the ion, namely, in the order Li > Na > K > Rb, where the retarding effect is greatest in the case of Li and least in the case of Rb; while in the case of monatomic monovalent anions the accelerating effect upon the rate of diffusion of positively charged particles of water from the side of pure water through the membrane into the solution increases directly with the radius of the ion I > Br > Cl; where I has the greatest and Cl the smallest attractive action.

This might be intelligible if the action of the ions on the particles of water were electrostatic, since in this case the action of the anion depends on the negative charge in its outermost shell of electrons and the electrostatic effect should be the greater the farther the shell is removed from the positive nucleus of the ion; while the electrostatic effect of the cation is due to the positive charge of the nucleus and this should be the greater the smaller the distance between nucleus and the outermost layer of electrons, *i.e.* the closer the positive nucleus can approach the water particles or the membrane particles on which the ion is to act.

<div style="text-align:center">IV.</div>

We have alluded to the fact that collodion membranes are not strictly semipermeable and that crystalline solutes diffuse out from the collodion flasks in our experiments. It might be argued that the differences in the flow of water measured in the preceding chapter are due to differences in the rate of diffusion of electrolytes from the side of the solution to the side of pure water through the collodion membrane. This assumption is, however, not tenable since it can be shown that the diffusion of the solutes into the pure water through the collodion membrane seems to follow Fick's diffusion law, according to which the rate of diffusion of a solute is directly proportional to its concentration and this seems to hold equally in the case of electrolytes and non-electrolytes. The specific influence of solutions of *electro-*

lytes on the rate of diffusion of water from pure water through collodion membranes into solutions can therefore not be due to any difference in the rate of diffusion of electrolytes and non-electrolytes through the membrane into the pure water, but must be ascribed to a difference in the behavior of water towards these two types of solutes.

<div align="center">V.</div>

We have thus far mentioned only the influence of electrolytes on the rate of diffusion of positively charged particles of water. Perrin found in his experiments on electrical endosmose that in certain cases the water migrated to the positive electrode, namely, when the solution had an acid reaction, while it migrated to the negative electrode when the solution had an alkaline reaction. No such reversal in the sign of electrification of water can be produced in the case of pure collodion membranes, since in this case the water is always positively charged no matter whether the solution is acid, neutral, or alkaline. When, however, we deposit a film of a protein on the inside (or on both sides) of the collodion membrane the latter becomes amphoteric. When the solution is sufficiently acid, the water migrates through the membrane as if its particles were negatively charged, while when the hydrogen ion concentration is lower, *i.e.*, when the solution is only very faintly acid or neutral or alkaline, the water particles move through the protein film of the membrane as if they were positively charged.

When we separate an acid solution of a salt by a collodion membrane possessing a protein film, from a solution of a pure acid of the same hydrogen ion concentration as that of the salt solution, the hydrogen ion concentration being equal to or above 10^{-4} N, the water migrates through the pores of the membrane as if its particles were negatively charged and as if they were "attracted" by the cation and "repelled" by the anion of the electrolyte in solution with a force increasing with the valency of the ion. In this case, water is "attracted" more powerfully by salts with trivalent cation, *e.g.*, AlCl$_3$ or LaCl$_3$, than by salts with bivalent cation *e.g.*, MgCl$_2$ or CaCl$_2$; and it is "attracted" more powerfully by the latter than by salts with monovalent cation, *e.g.*, NaCl or KCl; while negatively

charged water is not "attracted" by salts with bivalent or trivalent anions, e.g., Na_2SO_4 or Na_4 oxalate or $Na_4Fe(CN)_6$, etc.

In the case of salts with monatomic and monovalent cations the "attraction of" the salt for negatively charged water seems to increase inversely with the radius of the cation in the order Li > Na > K > Rb, where Li with the smallest radius "attracts" the negatively charged water most and Rb with the largest radius "attracts" the water least. The monatomic monovalent anions "repel" the negatively charged particles of water directly in proportion with the radius of the ion in the order I > Br > Cl, here I with the greatest radius "repels" the negatively charged water most, and Cl least.

The relative "attractive" and "repelling" action of the two oppositely charged ions of an electrolyte for negatively charged water is not the same in all concentrations. In the lowest concentrations the attractive influence of the cation for negatively charged water increases more rapidly with increasing concentration than does the repelling action of the anion; while beyond a certain concentration the repelling action of the anion on the negatively charged water increases more rapidly than the attractive action of the cation. Finally a concentration is reached where the electrical effects of the two oppositely charged ions balance each other more or less and from then on, the solution behaves more like that of a non-electrolyte.

VI.

In the course of these experiments facts were observed which indicate a chemical source for the electrification of water when in contact with a collodion membrane. We have mentioned the fact that when a membrane has been treated with a protein, the sign of the electrification of water in contact with the membrane can be reversed by acid. The protein forms a fine film on the surface and probably inside the pores of the collodion membrane. In an alkaline or neutral, and often even a very faintly acid concentration the water in contact with the protein film is positively charged, but when the hydrogen ion concentration exceeds a certain limit the water assumes a negative charge. The writer has measured the hydrogen ion concentration at which this reversal occurs and has found that it changes in a charac-

teristic way with a certain chemical constant of the protein which constitutes the film, namely, its isoelectric point.   Proteins are amphoteric electrolytes which behave differently on the two sides of a hydrogen ion concentration which is termed the isolectric point.   On the alkaline side from the isoclectric point proteins behave like a fatty acid, e.g., CH_3COOH, forming metal proteinates with alkalies, e.g., Na proteinate.   On the acid side of the isoelectric point the proteins behave like NH_3, forming protein-acid salts, e.g., protein chloride. We may imagine that proteins exist in the form of two isomers, one on the alkaline side of the isoelectric point possessing $COOH$ as the active chemical group; the other on the acid side of the isoelectric point possessing NH_2 as the chemically active group.   The isoelectric point, i.e., the hydrogen ion concentration at which the reversal of one type of protein salt to the other occurs, is a characteristic constitutional property of each protein.   Its value is, according to L. Michaelis, a hydrogen ion concentration of $10^{-4.7}$ N for gelatin and for casein, $10^{-4.8}$N for crystalline egg albumin, and $10^{-6.8}$N for oxyhemoglobin.

The writer has been able to show that the reversal of the sign of charge of water when in contact with a collodion membrane possessing a protein film practically coincides with the isoelectric point of the protein used, lying slightly on the acid side of this point.   The method of determining the hydrogen ion concentration at which the reversal in the sign of electrification of water occurs is as follows: We have shown that M/64 $CaCl_2$ or M/256 $LaCl_3$ "attracts" negatively charged water powerfully, while these two salts do not "attract" positively charged water.   On the other hand, Na_2SO_4 "attracts" positively charged water powerfully while it does not "attract" negatively charged water.   We fill a series of collodion bags previously treated with a protein each with a M/64 $CaCl_2$ solution, and dip each collodion bag into a beaker with distilled water.   The M/64 $CaCl_2$ solution in each bag is brought to a different hydrogen ion concentration by adding suitable quantities of HNO_3 or NaOH to the solution; and the distilled water in the beaker is always brought to the same hydrogen ion concentration as that of the M/64 $CaCl_2$ solution inside the collodion bag dipped into the beaker.   Similar experiments are made with Na_2SO_4 brought to a different hydrogen ion concentration.

The result of these experiments is striking. There is always one definite hydrogen ion concentration at which the "attraction" of both $M/64$ $CaCl_2$ (or $LaCl_3$) as well as that of $M/256$ Na_2SO_4 for water is almost zero. As soon as the hydrogen ion concentration rises, the attraction of $M/64$ $CaCl_2$ for water becomes noticeable and increases with a further increase in the hydrogen ion concentration until it reaches a maximum (at a hydrogen ion concentration of about $10^{-3}N$). The attraction of $M/256$ Na_2SO_4 for water rises when the hydrogen ion concentration falls below the point where the attraction is zero. $M/256$ Na_2SO_4 attracts water when it is positively charged and $M/64$ $CaCl_2$ does so when water is negatively charged. Where neither solution "attracts" water the latter is not electrified. (It should be mentioned that the attraction of a cane sugar solution of $M/64$ or below for water is very slight and scarcely

TABLE II.

Nature of Protein Film on the Membrane.	Hydrogen Ion Concentration where Water is Uncharged.		Isoelectric Point of Protein.
Gelatin........................	Between $10^{-4.0}$ and $10^{-4.6}$ N		$10^{-4.7}$ N
Casein........................	" " " "		$10^{-4.7}$ N
Egg albumin....................	" " " "		$10^{-4.8}$ N
Oxyhemoglobin.................	About $10^{-6.0}$ and $10^{-7.0}$ N		$10^{-6.8}$ N

noticeable, and that this is the reason that when water is not electrified it is not noticeably attracted by $M/64$ $CaCl_2$ or $M/256$ Na_2SO_4.) Table II shows the close relation of this hydrogen ion concentration and that of the isoelectric point for different proteins. Water begins to become negatively charged in contact with a collodion membrane as soon as the hydrogen ion concentration is slightly on the acid side of the isoelectric point of the protein forming a film on the membrane.

The quantitative agreement between the isoelectric point of the protein forming the film on a collodion membrane and the point of reversal of the sign of electrification of water is such that it is difficult to question the connection between the chemical constitution of the protein and the sign of electrification of water. It is also obvious that the density of the charge varies with the hydrogen ion concentration.

When the collodion membrane is not treated with a protein the water is always positively charged and no reversal in the sign of the charge can be obtained by an increase in the hydrogen ion concentration. This harmonizes with the fact that collodion is not an amphoteric electrolyte.

It is to be expected that in addition to the chemical nature of the membrane the chemical nature of the liquid in contact with the water also influences the sign (and density) of the electrical charge at the boundary of the two phases. Indications supporting this view exist but they can not be discussed in this connection.

<div align="center">VII.</div>

van't Hoff's theory of osmotic pressure confronted the physiologists with the puzzling fact that in the phenomena of secretion, water diffused often from places of higher to those of lower osmotic pressure. In 1908 Girard suggested that such cases of abnormal osmosis as occur in organisms might be explained on the assumption that the opposite sides of a membrane separating pure water from an acid or alkaline solution are oppositely charged, and that therefore Perrin's experiments on electrical endosmose furnish the explanation of these phenomena. According to Girard, only H or OH ions should produce such a difference in charge and neutral solutions of electrolytes should behave like solutions of non-electrolytes which is, however, not correct. Bernstein, in 1910, also reached the conclusion that electrical endosmose might be utilized for the explanation of abnormal osmosis as manifested in secretion, and in his book on "Electro-Biology" many speculations in this direction are offered but unfortunately very few experiments. He also assumes that the opposite sides of the membrane of a gland are oppositely charged. Under such circumstances positively charged water particles will be driven in the direction from the positive to the negative side of the membrane. As soon as the positively charged water particle reaches the negative side of the membrane it gives off its charge. This enables other positively charged water particles to follow.

Ideas similar to those offered by Girard and by Bernstein have been expressed by way of explanation of other cases of abnormal osmosis by Bartell and his collaborators, and by Freundlich.

Whatever the ultimate theory of the driving force in these cases may be, we have a right to state that the electrification of the particles of water migrating through a membrane is a fact; that the sign of this electrification seems to depend on the chemical nature of the membrane in contáct with water; that the rate of migration of these charged particles of water through the membrane from the side of pure water to the side of the solution is accelerated by the ions of the opposite sign of charge and retarded by the ions with the same sign of charge as that of the water with a force increasing with the valency of the ion; and that the relative acceleration and retarding effects of the two oppositely charged ions on the rate of diffusion of electrified water are not the same for all concentrations, that in lower concentrations of electrolytes the accelerating action of the oppositely charged ion increases at first more rapidly than the retarding effect of the other ion; while for higher concentrations the reverse is the case, until finally a concentration of the electrolyte is reached where the effects of the oppositely charged ions more nearly balance each other.

CHEMICAL AND PHYSICAL BEHAVIOR OF PROTEINS.[1]

BY JACQUES LOEB.

(From the Laboratories of The Rockfeller Institute for Medical Research.)

Life is so completely linked to the chemical and physical properties of proteins that the knowledge of these properties must precede the attempt at unraveling the dynamics of living matter. The modern concepts of colloid chemistry have been used to supply this knowledge, and foremost among these concepts is the idea that the reactions of colloids in general and proteins in particular are not determined by the purely chemical forces of primary valency, but by the rules of adsorption; and that the influence of electrolytes on the physical properties of proteins is due to an alteration in the degree of dispersion or in the degree of hydration of the protein particles. The writer has reached the conclusion that these views are based on a methodical error, so far as the proteins are concerned—namely, on the failure to take into consideration the hydrogen ion concentration, which happens to be the chief variable in the chemistry and physical chemistry of proteins. When this variable is duly considered, it is found that the laws of classical chemistry account for both the chemical and the physical behavior of the proteins.

Isoelectric Point.

Proteins are amphoteric electrolytes the chemical behavior of which depends on the hydrogen ion concentration of the solution. When the hydrogen ion concentration exceeds a certain critical value—which is termed the isoelectric point of the protein—the protein can combine only with anions, forming salts of the type of gelatin chloride, sulfate, etc., according to the nature of the acid added. When the hydrogen ion concentration is below this critical point, the protein can form metal proteinates, e.g., Na gelatinate, Ca gela

[1] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 39, 237, 363, 483, 559; 1920–21, iii, 85, 247, 391, 547, 557. *Science*, 1920, lii, 449.

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tinate, etc. At its isoelectric point, a protein can combine with neither anion nor cation. The natural method of freeing a protein from ionogenic impurities consists, therefore, in bringing the protein in powdered form to its isoelectric point and washing it with cold water.

Stoichiometrical Relationships.

The proof that the proteins combine by the purely chemical forces of primary valency and in strictly stoichiometrical proportions with acids and alkalies is furnished by the titration curves. If we add different acids to an isoelectric protein, we notice that in order to bring a protein solution to a definite pH—e.g., 3.0 or 2.5—exactly three times as many cc. are required of 0.1 N H_3PO_4 as of 0.1 N HCl or 0.1 N HNO_3; while equal numbers of cc. of 0.1 N H_2SO_4 and of HCl are required for this purpose. When we titrate with alkali, we find that exactly as many cc. of 0.1 N $Ca(OH)_2$ or of $Ba(OH)_2$ are required as of 0.1 N NaOH or KOH. These and many similar experiments leave no doubt that acids and alkalies combine with proteins according to the purely chemical forces of primary valency. The proof has been furnished not only for gelatin but also for crystalline egg albumin and for casein.

It follows from these experiments that the anions of weak dibasic or tribasic acids combine with proteins in the form of monovalent ions. Thus the anion of gelatin phosphate is the monovalent anion H_2PO_4 and not the trivalent anion PO_4; while in the case of gelatin sulfate the anion is the divalent SO_4 ion.

Properties a Function of Hydrogen Ion Concentration.

These facts furnish the clue for the understanding of the influence of ions on the physical properties of proteins. Before these experiments were made it was customary to express the effect of ions on the physical properties of proteins in terms of the so-called Hofmeister series. The writer has been able to show that these series are based on an error due to the fact that the influence of the variation in the hydrogen ion concentration was overlooked and erroneously interpreted as being the expression of a specific influence of the anion of the acid. If the effect of different ions is compared at the same

hydrogen ion concentration, it is found that only the valency of the ion with which the protein is in combination influences the properties of a protein; while the nature of the ion is of no significance. Thus gelatin chloride, nitrate, tartrate, succinate, citrate, and phosphate have the same osmotic pressure, the same viscosity, the same amount of swelling (at the same hydrogen ion concentration and the same concentration of originally isoelectric gelatin), for the reason that in all these cases the anion with which the protein is in combination is monovalent. The osmotic pressure, swelling, and viscosity of gelatin sulfate are, however, considerably lower than those of gelatin chloride of the same pH and the same concentration of gelatin, for the reason that the SO_4 ion in combination with the gelatin is bivalent.

The fact that only the valency of an ion influences the physical properties of a protein, while the nature of the ion is of no significance (unless it causes a constitutional change in the protein molecule), is the crucial point in the interpretation of the influence of ions on the physical properties of proteins; since this fact suggests that this influence depends on simple equilibrium conditions and not on a change in the protein, such as hydration or degree of dispersion.

When increasing quantities of the same acid are added to isoelectric protein—e.g., a 1 per cent solution of isoelectric gelatin—the values for osmotic pressure, swelling, viscosity, first increase until a a maximum is reached (at a pH varying for the three properties between 3.6 and 3.0) and drop again with a further increase in acid added (Fig. 1). Colloid chemistry explains such variations on the assumption of variations in the degree of aggregation of the particles or of variations in the degree of hydration of the protein particles. When a neutral salt is added to a protein solution, the osmotic pressure, swelling, and viscosity drop, and this is also ascribed to a depression in the degree of dispersion or hydration of the protein particles.

The Donnan Equilibrium.

Recent investigations have led the writer to a different view—namely, that the influence of valency, hydrogen ion concentration, and the addition of salt on the physical properties of proteins find their explanation in a field entirely foreign to colloid chemistry—

namely, in a peculiar equilibrium condition the theory of which was worked out by Donnan. Donnan[2] has shown that when we separate two salt solutions by a membrane which is impermeable for one of the ions while permeable for all the rest, a peculiar equilibrium results at which the distribution of the ions on the opposite sides of the
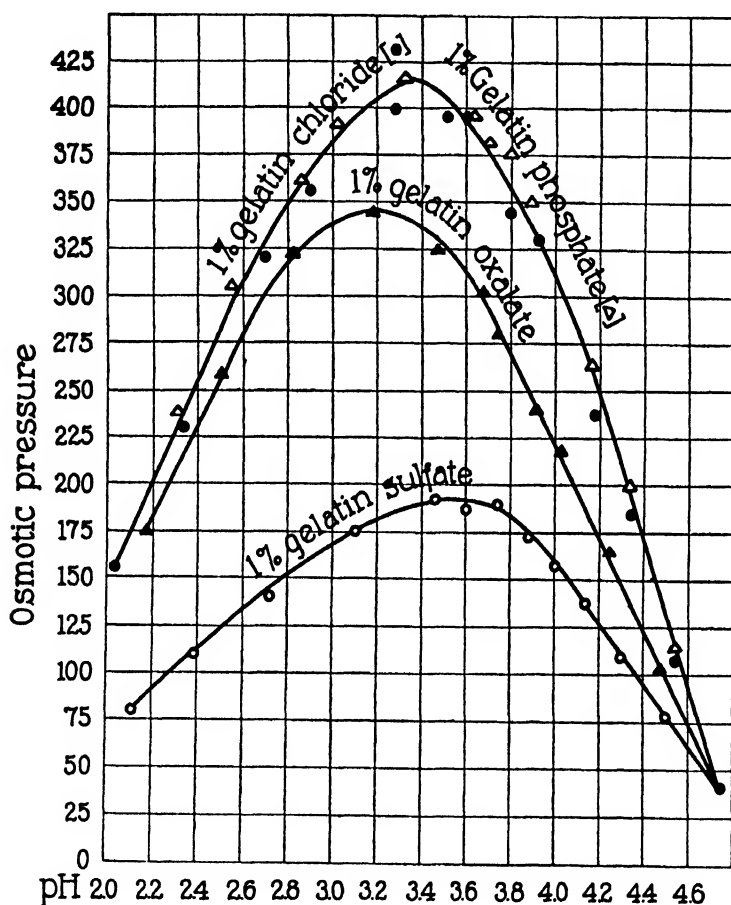
FIG. 1. Observed variation of osmotic pressure with hydrogen ion concentration.

membrane is unequal. It is immaterial whether the ion which cannot diffuse through the membrane is a colloid or a crystalloid. Proc-

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[2] Donnan, F. G., *Z. Elektrochem.*, 1911, xvii, 572. Donnan, F. G., and Harris, A. B., *J. Chem. Soc.*, 1911, xcix, 1554. Donnan, F. G., and Garner, W. E., *J. Chem. Soc.*, 1919, cxv, 1313.

ter[3] made use of this Donnan equilibrium to prove that the swelling of solid gelatin chloride is an osmotic phenomenon which can be explained quantitatively on the basis of the Donnan equilibrium.

In our investigations we started with a measurement of the potential differences which exist between a 1 per cent gelatin chloride solution contained in a collodion bag and the surrounding water after osmotic equilibrium is established (*e.g.*, after 18 hours). It was found that the influence of the hydrogen ion concentration, the valency of the anion in combination with the gelatin, and the addition of neutral salt on the P.D. was similar to the influence of the three factors on the osmotic pressure, the swelling, and the viscosity of proteins. This in itself would have meant little more than that a fourth property of proteins had been found which varies like the other three properties mentioned, if it had not been for the fact that it was possible to correlate the variations of the P.D. quantitatively with the Donnan equilibrium.

When a gelatin chloride solution is separated from pure water by a collodion membrane, free HCl diffuses into the water, and when equilibrium is established the concentration of acid in the water (the "outside solution") is greater than in the gelatin solution (the inside solution). This was observed by Procter for blocks of gelatin chloride surrounded by water, and by the writer when a solution of gelatin chloride was separated from water by a collodion membrane. Procter has shown that on the basis of Donnan's theory the equilibrium is defined by the equation $x^2 = y(y + z)$, where x is the concentration of the H and Cl ions in the outside solution, y the concentration of the H and Cl ions of the free acid in the gelatin (inside) solution, and z the number of Cl ions in combination with gelatin.

If we write the equation in the form

$$\frac{y}{x} = \frac{x}{y+z}$$

$\frac{y}{x}$ becomes the ratio of the concentration of the hydrogen ions inside over those outside. If we assume that the P.D. measured in our experi-

[3] Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313. Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

ments was due to the difference in the hydrogen ion concentration on the opposite sides of the membrane, then it would follow from Nernst's well-known logarithmic formula that the P.D. should be equal to $0.058 \log \frac{y}{x}$. Log y is, however, the pH of the inside solution and log x is the pH of the outside solution. Hence, if the Donnan equilibrium was responsible for the P.D., the observed P.D. should be equal 0.058 (pH inside minus pH outside).

Calculated and Observed Potential Differences.

It was found that the values for the P.D. calculated on this assumption were in good agreement with the observed values (Tables I,

TABLE I.

pH Inside Minus pH Outside after 18 Hours.

Original inside solution, 1 per cent originally isoelectric gelatin dissolved in various concentrations of NaNO₃ made up with HCl to pH 3.5.

Outside solution, same concentrations of NaNO₃ all made up with HCl to pH 3.0.

	Concentration of NaNO₃.								
	0	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32
pH of inside solution.......	3.58	3.56	3.51	3.46	3.41	3.36	3.32	3.29	3.25
" " outside "	3.05	3.08	3.10	3.11	3.14	3.17	3.20	3.22	3.24
Difference pH inside minus pH outside.............	0.53	0.48	0.41	0.35	0.27	0.19	0.12	0.07	0.01

II, and III). This left no doubt that the influence of the valency, of the hydrogen ion concentration, and of the addition of neutral salt on the variation of the P.D. found its complete qualitative and quantitative explanation on the basis of the Donnan equilibrium.

The close analogy between the influence of pH, valency, and neutral salt on the P.D., and on osmotic pressure and swelling, suggested the possibility that these latter properties might also be determined by the Donnan equilibrium. We have calculated on the basis of the Donnan theory the influence of the valency of the anion

and of the pH on the osmotic pressure of 1 per cent solutions of gelatin phosphate, chloride, and sulfate, and of albumin chloride and sulfate, and find that the calculated values agree with the observed osmotic pressures not only qualitatively but practically quantitatively. Thus the curves of the observed osmotic pressure of gelatin chloride and gelatin phosphate (Fig. 1) when plotted over the pH as abscissae are practically identical and so are the curves calculated on the basis of Donnan's theory (Fig. 2); and, what is more, the calculated and observed curves are, except for two minor variations, practically identical. Furthermore, the observed osmotic pressures for gelatin sulfate are not quite one-half of the observed pressures

TABLE II.

Potential Difference between Gelatin Solution and Outside Solution.

Concentration of NaNO₃.	Calculated by Nernst's formula from pH.	Observed.
	millivolts	*millivolts*
0	31.2	31
M/4,096	28.3	28
M/2,048	24.0	24
M/1,024	20.7	22
M/512	16.0	16
M/256	11.2	12
M/128	7.0	7
M/64	4.1	4
M/32	0	0

for gelatin chloride of the same pH and concentration of originally isoelectric gelatin (Fig. 1). The same difference exists between the calculated osmotic pressures for gelatin chloride and gelatin sulfate (Fig. 2).

Nothing brings out more clearly the difference between the viewpoint of colloid chemistry and the viewpoint of classical physical chemistry than this latter result. In the literature of colloid chemistry SO_4 is called a dehydrating ion which is supposed to diminish the swelling and the osmotic pressure of proteins through a modification of the protein. From our viewpoint the apparent dehydrating effect of SO_4 is merely the consequence of the fact that the valency of the

TABLE III.

Influence of the Hydrogen Ion Concentration on pH Inside Minus pH Outside and on the P. D. of Gelatin Chloride Solutions at Equilibrium.

	Cc. 0 1 N HCl added to 100 cc. 1 per cent isoelectric gelatin.											
	1	2	4;	6	8	10	12 5	15	20	30	40	50
pH inside..........	4.56	4.31	4.03	3.85	3.33	3.25	2.85	2.52	2.13	1.99	1.79	1.57
pH outside.........	4.14	3.78	3.44	3.26	2.87	2.81	2.53	2.28	2.00	1.89	1.72	1.53
pH inside minus pH outside......	0.42	0.53	0.59	0.59	0.46	0.44	0.32	0.24	0.13	0.10	0.07	0.04
P.D. calculated (millivolts)......	+24.7	+31.0	+34.5	+34.5	+27.0	+25.8	+18.8	+14.0	+ 7.6	+5.9	+4.1	+2.3
P.D. observed (millivolts).......	+24.0	+32.0	+33.0	+32.5	+26.0	+24.5	+16.5	+11.2	+ 6.4	+4.8	+3.7	+2.1

ion with which the protein is in combination modifies the relative distribution of the crystalloidal ions on the two sides of the membrane.
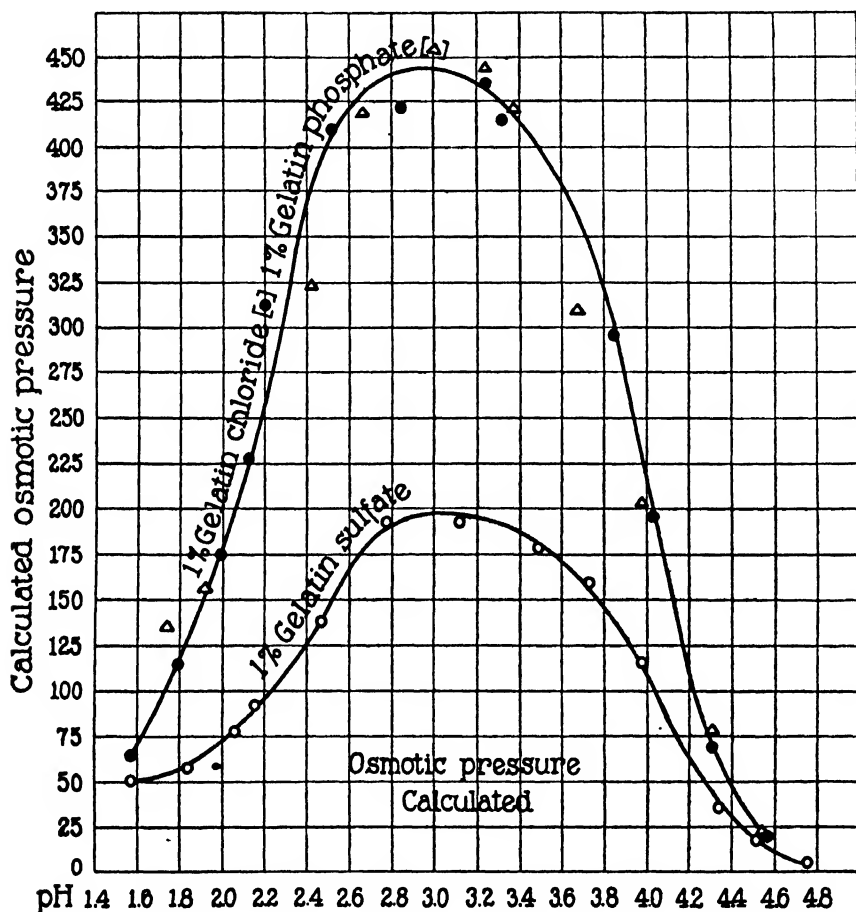
FIG. 2. Calculated variation of osmotic pressure with hydrogen ion concentration.

The fact that the Donnan equilibrium is the basis of the variation in osmotic pressure explains also why only the valency and not the nature of the ion has any influence on the osmotic pressure, since the equilibrium equation is the same for all protein-acid salts with monovalent anion. The nature of the anion does not enter into the equilibrium equation.

Quantitative Theory in Development.

If we summarize all these results, we may say that the experiments based on the measurement of the hydrogen ion concentration have proved that the proteins combine with acids and alkalies according to the purely chemical forces of primary valency and in the same stoichiometrical relations in which acids and alkalies combine with crystalloids. They have led to the result that only the valency but not the nature of the ion in combination with a protein affects such physical properties as the P.D. and osmotic pressure and they have further led to the result that this fact finds its explanation in the Donnan membrane equilibrium. Moreover, it was possible to show that the influence of the hydrogen ion concentration on the P.D. and on the osmotic pressure of protein solutions can also be accounted for not only qualitatively but almost quantitatively by Donnan's theory of membrane equilibrium. Procter's experiments and some of the writer's experiments which are not yet complete suggest that the influence of the hydrogen ion concentration and of the valency of the anion on the swelling of gelatin-acid salts may possibly be explained in the same way. The classical laws of general and physical chemistry therefore furnish us with a quantitative theory not only of the chemical behavior of proteins but also of at least some of the physical properties.

EXPERIMENTAL STUDIES IN DIABETES.

SERIES II. THE INTERNAL PANCREATIC FUNCTION IN RELATION TO BODY MASS AND METABOLISM.

1. ALTERATIONS OF CARBOHYDRATE ASSIMILATION BY REMOVAL OF PORTIONS OF THE PANCREAS.

BY FREDERICK M. ALLEN, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research, New York.*)

The preceding series of papers[1] gave a general outline of the checking of diabetes by fasting and restriction of total diet on the current theory of sparing the weakened pancreatic function. This speculative clinical hypothesis thus received positive physiologic and anatomic demonstration, so that the possibility of damaging the islands of Langerhans by functional overstrain and saving them by limiting the dietary burden stands as an established fact. At the same time the vagueness of this language is unfortunate and more information is desirable concerning the nature of the island function and the processes involved in overtaxing and resting it. This need is both theoretical, for understanding the endocrine role of the pancreas, and practical, for the most accurate and effective application of treatment. The same observations also afford the opportunity for such a study by furnishing plain physiologic and anatomic criteria by which the effect of various influences upon the islands can be judged.

As shown elsewhere, an explanation of the effect of fasting is not found in any gross or microscopic alterations in the pancreas; in particular there is no evidence of an increase of islands by fasting. The changes in question must be sought elsewhere in the body. The known effects of fasting and low diet are a reduction of body weight and metabolism. Each of these may be subdivided.

(*a*) The reduction of weight may pertain either to storage materials or to active protoplasm. It may seem natural that a reduction of stored foods should increase the capacity of the body to receive food. As a matter of fact no such rule has been demonstrable for the normal organism, particularly with reference to the most easily excreted

[1] Allen, F. M.: Jour. Exper. Med., 1920, vol. xxxi.

food, carbohydrate. The utmost glycogen storage does not lower the sugar tolerance. On the contrary a dose of glucose improves the assimilation of a closely succeeding dose.[2] Though respiration studies indicate that fasting creates a tendency to storage of carbohydrate as opposed to combustion,[3] the tendency to hyperglycemia and glycosuria is notoriously increased by either fasting or carbohydrate-free diet.[4] Evidently some state of preparedness of the organism is here more determining than the mere fulness or emptiness of the depots.

(b) A reduction of active protoplasm may receive two opposite interpretations. On the one hand it may be supposed that reduction of the living matter which carries on metabolism will involve a reduced capacity for metabolism On the other hand a quantitative relation may be conceived between the mass of pancreas and the mass of tissue which it can supply. As the pancreas of a mouse could not possibly suffice for the body of an elephant, the same principle may hold for less extreme differences; and when the pancreatic hormone is deficient the relatively greater supply resulting from a diminution of protoplasm may facilitate metabolism.

(c) Metabolism likewise may be variously regarded in this connection. Distinctions may be drawn between the metabolism of matter and of energy, and in the broadest sense between the exogenous or external activities of the cells and their endogenous or structural metabolism. The relation of the pancreatic function to the three chief food substances, carbohydrate, protein and fat, is also involved. In addition the influence of the other endocrine organs which affect metabolism requires consideration.

All the above relations are capable of experimental modification in various ways. Having the above criteria of pancreatic function,

[2] Hamman, L., and Hirschman, I. I.: Bull. Johns Hopkins Hosp., 1919, xxx, 306.

[3] Johansson, J. E., Billström, J., and Heijl, C.: Skand. Arch. Physiol., 1904, xvi, 263–272. Johansson: Ibid., 1909, xxi, 1–34. Falta, W.: Med. Klin., 1914, x, 9.

[4] Hofmeister, F.: Arch. exp. Path. u. Pharm., 1889–90, xxvi, 355. Allen, F. M.: Glycosuria and Diabetes, 1913, Chapter XIII; Jour. Exp. Med., 1920, xxxi, 557.

it is a simple matter to perform a series of experiments to furnish some information concerning this function. One group of such experiments was planned to investigate the quantitative relations of this function with the body mass and metabolism, the latter two being viewed theoretically as separate though never strictly separable in practice. Intimately connected with this problem are questions of the nature of the function: whether the quantitative relation is with food, so that a certain quantity of the internal secretion is necessary for the utilization of a certain quantity of food, or with the cells, so that they require a certain minimum quantity of the secretion, and if possessing it can metabolize indefinite quantities of food; whether the function pertains primarily to carbohydrate alone or also to protein and fat; and other questions of this character concerning the mechanism of the internal pancreatic secretion and its place in metabolism. As far as possible the experiments dealing with quantitative relations between the pancreas and the occurrence of diabetes or certain characteristic diabetic phenomena have been grouped in the present series, of which papers 1 to 4 are published in this JOURNAL and papers 5 to 12 in the *American Journal of Physiology*. Other series pertaining to the nature of the endocrine pancreatic function are presented in other journals.

The most direct means of altering the relation between pancreas mass and body mass is by surgical resections. The first experiments of the series deal with the removal of various portions of the pancreas and observations of the tolerance for Merck's anhydrous glucose by stomach and by subcutaneous injection in 30 per cent. solution. The usual precautions and standardized conditions were employed.* Unless otherwise specified the diet was bread and soup *ad libitum* and the tests were performed after twenty-four hours' fasting. The urinary sugar was determined by titration with Benedict's solution and the blood sugar by the method of Lewis and Benedict.

REMARKS ON DOG B2–00.

1. The above dog received various doses of Merck glucose by stomach tube and subcutaneously after removal of successive portions

* All operations were performed under ether anesthesia.

TABLE I.

Dog B2–00.

Weight 14 Kgm.

Date, 1913	Glucose dosage, gm. per kgm.	Hours following injection.	Glycosuria, gm.	Plasma sugar, per cent.	Remarks.
Nov. 1	8.0	24	Faint	..	Subcutaneous injection; normal dog.
8	6.0	24	Slight	..	Subcutaneous injection.
13	2.0	24	0	..	Subcutaneous injection.
17	7.0	24	0	..	Stomach tube.
20	7.0	24	0	..	Subcutaneous injection.
29	9.0	24	0	..	Subcutaneous injection.
Dec. 8	Removed pancreatic tissue weighing 7.1 gm., consisting of most of the splenic end.				
20	6.0	6	0.4	..	Subcutaneous injection.
		24	Faint		
30	4.5	24	0.3	..	Subcutaneous injection.
1914.	.				
Jan. 2	3.0	24	0.2	..	Subcutaneous injection.
5	2.5	24	0.2	..	Subcutaneous injection.
9	1.8	24	0	..	Subcutaneous injection.
13	4.0	1½	Faint	..	Stomach tube.
		3	Faint	..	
		24	0		
Jan. 19	Removed processus uncinatus weighing 4 gm.				
Feb. 5	2.0	5½	Faint	..	Subcutaneous injection.
		24	0		
11	1½	4	Slight	..	Subcutaneous injection.
		24	Faint		
Mar. 26	1½	6	Faint	..	Subcutaneous injection.
		24	0		
April 3	2.1	24	Faint	..	Subcutaneous injection.
20	2.4	6	Faint	..	Subcutaneous injection; this injection given two hours after feeding.
		24	0		
27	2.4	6	Faint	.f.	Subcutaneous injection.
		24	0		
May 19	2.7	4	Faint	..	Subcutaneous injection.
		24	0		
27	3.0	7½	Faint	..	Subcutaneous injection.
		24	0		
June 2	4.0	24	Faint	..	Subcutaneous injection.
8	5.0	24	Faint	..	Subcutaneous injection.
26	6.0	24	Negative	..	Subcutaneous injection.
July 2	6.0	1½	0	..	Stomach tube.
		3½	0		
		24	0		
8	7.0	2	Faint	..	Subcutaneous injection.
		24	0.34		
30	7.0	3	0	..	Stomach tube.
		24	0		
Nov. 9	7.0	2½	Slight	..	Subcutaneous injection.
		24	Slight		

TABLE I—*Concluded.*

Date, 1914	Glucose dosage, gm. per kgm.	Hours following injection.	Glycosuria, gm.	Plasma sugar, per cent.	Remarks.
Dec. 10		Removed stump of the processus lienalis and adjoining part of corpus, weighing 6.3 gm.			
1915.					
Mar. 16	6.0	24	0	..	Subcutaneous injection.
June 2	3.0	3	Slight	..	Subcutaneous injection.
		8	0 69		
		12	0.82		
		24	Faint		
11	1.5	4	0	..	Subcutaneous injection.
		8½	0		
		10	0		
		24	0		
18	3.0	1	0	..	Stomach tube.
		7	0		
		9	0		
		24	0		
30	4.0	22½	0	..	Subcutaneous injection.
1916.					
Sept. 6		Removed 0.8 gm. pancreatic tissue.			
Nov. 15	4.0	2	0.26	..	Stomach tube.
		3½	0		
20		Before feeding	0	0.102	Fed 200 gm. bread.
		2	0	0 125	100 gm. lung.
		4	0.36	0.154	75 gm. glucose.
		6	0	0.149	
21		Before feeding	0	0.106	Fed 200 gm. bread
		2	0	0.123	100 gm. lung.
		4	Faint	0.156	150 gm. glucose.
		6	Faint	0 128	
23		Before feeding	0	0.092	Fed 200 gm. bread.
		2	0	0 095	100 gm. lung.
		4	0	0.139	150 gm. glucose.
		6	0	0 130	
Dec. 16		Removed 0.1 gm. of pancreatic tissue. Dog pregnant.			
30		Before feeding	0	0 085	Fed 200 gm. bread.
		2	3.45	0.345	100 gm. lung.
		4	6.46	0.455	150 gm. glucose.
		6	5.89	0.500	
1917.					
Jan. 7	..	Before feeding	0	0.125	Fed 200 gm. bread.
		2	7.13	0.400	100 gm. lung.
		4	6.68	0.400	150 gm. glucose.
		6	8.0	0.333	
Feb. 23		Before feeding	0	0.116	Fed 200 gm. bread.
		2	0.63	0.238	100 gm. lung.
		4	0.57	0.244	150 gm. glucose.
		6	0.69	0.232	

of the pancreas, the standard of tolerance being the smallest quantity with which distinct glycosuria could be produced. The first removal of 7.1 gm. from the splenic end, representing between a fourth and a third of the pancreas, resulted in a perceptible lowering of tolerance by this test, so that glycosuria resulted from the injection of as little as 2.5 gm. per kgm. when it had formerly been absent with as much as 9 gm. per kgm. Further reductions of the tolerance followed the removal of further portions of the pancreas.

2. There are irregularities in individual tests. For example, the slight reaction with 6 gm. per kgm. on November 8, 1913, is an instance of accidental variation, and it is probable that the dog was slightly nervous during the first two tests. On January 5, 1914, there was glycosuria with 2.5 gm. per kgm. and in February and March with 1.5 gm. per kgm., while on June 26 and the ensuing March 16, after removal of more pancreatic tissue, as much as 6 gm. per kgm. was tolerated. These discrepancies with subcutaneous injection were paralleled by those found with the stomach tube administration. Possibly a real recovery of pancreatic function during the long period of time may be the reason. In general the decline of tolerance is evident as mentioned.

· 3. As the verge of diabetes was approached small fragments of pancreatic tissue acquired great importance. Prolonged carbo-hydrate-rich diet (June 30, 1915, to September 6, 1916) did not bring on diabetes. On the latter date the tolerance was perceptibly lowered by the removal of 0.8 gm. pancreatic tissue, which could never have been missed if removed from a normal animal. Diabetes was caused by the removal of only 0.1 gm. of tissue on December 16. Some influence may be attributed to the attendant inflammatory injury, but the inflammation produced by the careful removal of such a small bit of tissue is very slight.

4. Blood-sugar analyses were impossible at first but were intro-duced as soon as facilities permitted. They serve for little more than rough indication of the height and duration of hyperglycemia and to exclude any extraordinary slowness of absorption or variations of renal permeability. With the increasing delicacy of tests necessary at this stage, factors of this sort assumed such importance that recourse was had to intravenous tests in other animals, as described

elsewhere.[5]   The methods employed for the present animals were discontinued and for other reasons some feeding experiments were performed, which are appended for their interest in the present connection.   The complication introduced by pregnancy is discussed in a subsequent paper (No. 9 of this series).

5. These feeding experiments, from November 15 to November 23, 1916, show that although the apparent tolerance heretofore has been lowered so that glycosuria resulted from 4 gm. or less of glucose per kgm., the actual tolerance was so high that only moderate hyperglycemia and trivial glycosuria resulted from as much as 100 gm. beef lung, 200 gm. bread and 150 gm. glucose.*   As a matter of fact the attempt to produce continuous glycosuria or lowering of assimilation by the highest and most prolonged possible feeding of bread and glucose failed and the tolerance actually rose as indicated by the results in blood and urine on November 23 in comparison with the preceding days.   The removal of the tiny fragment of pancreatic tissue on December 16 produced a decisive change, as shown in two ways: (1) The difference in hyperglycemia and glycosuria on the identical diet is striking and on a different plane from anything following the former operations; (2) the tendency to loss of tolerance was progressive.   Slight glycosuria resulted from plain bread and soup diet, so that the diet had to be changed to beef lung.   With this sparing of the function and termination of the pregnancy, improvement of assimilation was possible.   Such progressive improvement was shown in the tests from December 30, 1916, to February 23, 1917, in Table I, and still more in the later ones reported in paper 3 of Series I.   As there recorded the identical diet on August 9 and October 5, 1917, gave results in blood and urine practically the same as those in November, 1916.   The great actual difference in condition is shown by the later history of the dog as given in the paper mentioned, for the mere prolonged continuance of a high-calory carbohydrate-free diet gradually broke down the tolerance.   An

[5] Allen, F. M., and Wishart, Mary B.: Jour. Biol. Chem., 1920, xlii, 415.

* The distinction between these traces of glycosuria and the genuine limits of assimilation is illustrated further by the test of April 20, 1914, when the assimilation of 6 gm. glucose per kgm. subcutaneously was not perceptibly lowered by giving it two hours after the dog had eaten her fill of bread and soup.

agreement of physiologic and anatomic evidence thus indicates the excessive labor of an inadequate pancreas remnant in the attempt to prove equal to its task and its gradual breakdown under the strain.

This observation harmonizes with that made with the glucose tests under (2) above, where there was an apparent rise of tolerance, though the animal after removal of more pancreatic tissue must have been closer to diabetes. The conclusion must be that though some approximate information is derivable from such assimilation tests, neither the administration of glucose by stomach or subcutaneously nor the feeding of a mixed meal is necessarily decisive concerning the precise state of an animal as respects diabetes.

<div style="text-align:center">REMARKS ON DOG B2–01.</div>

Essentially the same points are noted as in Dog B2–00. The largest subcutaneous dose which the normal animal assimilated without glycosuria was 7 gm. per kilo. After removal of only the splenic process, glycosuria resulted from 3 gm., but not from 2.5 gm. per kilo. After removal of the uncinate process there was glycosuria with 1.5 gm. per kilo.

· The retention of actual assimilative power was shown on April 20, 1914, when the tolerance for 2.4 gm. glucose per kilo was scarcely altered (as compared with April 27) by a full meal of bread and soup four hours before the subcutaneous injection. After removal of 7 gm. additional pancreatic tissue there was a tendency to a rise of the apparent tolerance, as indicated by the trivial glycosuria from 4 gm. glucose per kilo on June 8 and July 13; but the dog was approaching the border of diabetes, as shown by the marked effect on removing 1.25 gm. tissue on November 24. Nevertheless, prolonged high starch and glucose diets were unable to produce any continued glycosuria or lowering of tolerance even with removal of 0.85 gm. additional tissue on April 26, 1916, so that diabetes actually resulted only with the removal of 0.8 gm. tissue on August 31, 1916. This operation caused no immediate striking change in the condition. A lowering of tolerance was indicated by the greater glycosuria from 4 gm. glucose per kilo on November 15 than in the last preceding test by stomach tube. But large carbohydrate meals were still almost completely

TABLE II.

Dog B2–01.

Weight 14 kgm.

Date.	Glucose dosage, gm. per kgm.	Hours following injection.	Plasma sugar, per cent.	Glycosuria, gm.	Remarks.
1913					
Nov. 1	8.0	24	..	Slight.	Subcutaneous injection; normal dog.
11	4.0	24	..	0	Subcutaneous injection; normal dog.
16	8.0	2	..	1.30	Stomach tube.
20	7.0	5	..	0	Subcutaneous injection.
		24	..	0	
29	9.0	6	..	Slight	Subcutaneous injection.
		24	..	0	
Dec. 6	8.0	5½	..	0.60	Subcutaneous injection.
		24	..	Slight	
12	Removed processus lienalis, weighing 8 gm.; estimated as little less than one-third pancreas.				
21	4.5	6	..	0	Subcutaneous injection.
		24	..	Slight	
30	4.5	24	..	0 40	Subcutaneous injection.
1914					
Jan. 2	3 0	24	..	0.80	Subcutaneous injection.
5	2.5	24	..	0	Subcutaneous injection.
13	4 0	1	..	Faint	Subcutaneous injection.
		2	..	0	
		24	..	0	
20	Removed processus uncinatus of pancreas, weighing 5.9 gm.				
Feb. 5	2.0	5	..	0.12	Subcutaneous injection.
		24	..	0	
11	1.5	4	..	Slight	Subcutaneous injection.
		24	..	Faint	
Mar. 26	1.5	6	..	Faint	Subcutaneous injection.
		24	..	0	
April 3	2.1	24	..	Faint	Subcutaneous injection.
20	2.4	6	..	Slight	Subcutaneous injection; fed bread and soup four hours before test.
		24	..	0	
27	2.4	6	..	0	Subcutaneous injection.
		24	..	0	
May 15	Removed splenic end of body, weighing 7 gm.				
June 1	2.0	24	..	Faint	Subcutaneous injection.
8	4.0	24	..	Faint	Subcutaneous injection.
July 9	6.0	2	..	0.72 (2.40%)	Stomach tube.
		24	..	0	

TABLE II—*Concluded.*

Date.	Glucose dosage, gm. per kgm.	Hours following injection.	Plasma sugar, per cent.	Glycosuria, gm.	Remarks.
1914					
July 13	4.0	2	..	Faint	Stomach tube.
		24	..	0	
17	7.0	2	..	0.27 (2.70%)	Subcutaneous injection.
		24	..	Slight	
Nov. 9	7.0	2	..	1.20 (5.00%)	Subcutaneous injection.
		24	..	1.20 (2.00%)	
24	Removed pancreatic tissue weighing 1.25 gm.				
1915					
April 30	4.0	Before feeding	0.107	0	Stomach tube.
		½	0.244	0.03 (0.25%)	
		1	0.250	0 07 (2.27%)	
		2¼	0.266	0 45 (5.50%)	
		4¼	..	0 92 (7.10%)	
		6	0.184	1.00 (8.30%)	
		8	0.324	0 23 (2.40%)	
		10	0.230	0.11 (0.70%)	
		12	..	Faint	
		13	0.186	Faint	
		15	0.163	Faint	
		24	0.146	0	
Sept. 29	4.0	Before feeding	0.106	0	
		1½	·0.256	Slight	Stomach tube.
		3½	0.147	Faint	
		7	0.103	0	
1916					
April 26	Removed pancreatic tissue weighing 0.85 gm.				
Aug. 31	Removed pancreatic tissue weighing 0.80 gm.				
Nov. 15		2	..	1.10 (6.30%)	Stomach tube.
	4.0	5½	..	1.70 (4.60%)	
		24	..	0	

assimilated and some power of regaining tolerance remained, as indicated by the lower plasma sugars on February 23, 1917, as compared with November 21, 1916. (See record in following paper.) Nevertheless, the downward tendency continued into severe diabetes, as described in the later history.

REMARKS ON DOG B2–02.

The observations resemble those in the two preceding dogs. The dog tolerated 7 gm. glucose per kilo subcutaneously and as much as

TABLE III.

Dog B2–02.

Weight 10.5 Kgm.

Date.	Glucose dosage, gm. per kgm.	Hours following injection.	Glycosuria. gm.	Remarks.
1913				
Nov. 1	8.0	24	Slight	Subcutaneous injection; normal dog.
11	4.0	24	Slight	Subcutaneous injection.
15	8.0	1	Faint	Stomach tube.
		24	0	
20	7.0	5	0	Subcutaneous injection.
		24	0	
22	10.0	4	0	Stomach tube.
		24	0	
29	9.0	6½	0.4	Subcutaneous injection.
		24	0	
Dec. 6	8.0	5	0.2	Subcutaneous injection.
		24	Slight	
9	Removed processus uncinatus of the pancreas, weighing 6.8 gm.			
22	5.0	6	1 4	Subcutaneous injection.
		24	Slight	
30	3.5	24	Negative	Subcutaneous injection.
1914				
Jan. 2	4.0	24	Slight	Subcutaneous injection.
13	4.0	1¾	Faint	Stomach tube.
		3	0	
		24	0	
20	4.0	5½	Slight	Subcutaneous injection.
		24	0	
23	Removed processus lienalis of the pancreas, weighing 9.7 gm.			
Feb. 5	2.0	5	0.1	Subcutaneous injection.
		24	0	
11	1.5	3½	Slight	Subcutaneous injection.
		24	0	
Mar. 26	1.5	5½	0	Subcutaneous injection.
April 3	2.1	24	Doubtful	Subcutaneous injection.
20	2.4	5½	0	Subcutaneous injection; fed bread and soup three hours before injection.
		24	0	
27	3.0	6¼	Faint	Subcutaneous injection.
		24	0	
May 19	3.2	4	Faint	Subcutaneous injection.
		24	Faint	
27	4.0	7	Faint	Subcutaneous injection.
		24	0	
June 2	4.0	24	Faint	Subcutaneous injection.
8	5.0	24	Faint	Subcutaneous injection.
26	6.0	24	0	Subcutaneous injection.

TABLE III—*Concluded.*

Date.	Glucose dosage, gm. per kgm.	Hours following injection.	Glycosuria, gm.	Remarks.
1914				
July 2	6.0	2½	0.6	Stomach tube.
		4	0	
		24	0	
7	4.0	3	0.9	Stomach tube.
		24	0	
13	3.0	2	0	Stomach tube.
		24	0	
17	7.0	1½	1.8	Subcutaneous injection.
		24	Faint	
Nov. 9	6.0	2	0 5	Subcutaneous injection.
		24	Slight	
Dec. 10	Removed pancreatic tissue weighing 4.75 gm.			
1915				
June 2	3.0	24	Faint	Subcutaneous injection.
11	2.0	24	0	Subcutaneous injection.
18	3.0	24	0	Stomach tube.
30	4.0	20	0.8	Subcutaneous injection.

10 gm. per kilo by stomach tube.   After the first operation the apparent tolerance subcutaneously lay between 3.5 and 4 gm. per kilo. Thus the removal of the uncinate process was as effective as the removal of the splenic process in the preceding dogs, and it may be remarked that other tests in other dogs have shown no appreciable differences in endocrine potency between different portions of the pancreas.   After removal of the splenic process on January 23, 1914, the tolerance fell further so as to lie between 2 and 3 gm. per kilo, but fluctuated somewhat, and as usually the glycosuria, even with much higher dosages, was trivial.   Very mild diabetes was produced by the third operation on December 10, 1914.   Special mention may be made of two points in this connection: (1) No tests were performed until the following June, when the usual effort at recuperation was evidently in progress, for the apparent tolerance had risen to about 3 gm. per kgm.; (2) the operation to produce diabetes took place much earlier in this dog than in the two preceding and the diabetes occurred with fully as large a pancreas remnant.   This animal therefore serves as a control to the other two, to show that the

much longer carbohydrate feeding to which they were subjected was not a factor in the production of their diabetes.

The subsequent history of this dog was given in paper 2 of Series I. The evidence of the existence of diabetes was that glycosuria could be readily maintained at any time by the addition of glucose to the diet. The dog would never take glucose long without vomiting, and would eat only moderate quantities of bread, so that she remained free from glycosuria until death from rabies on September 19, 1916. The islands of Langerhans then showed slight vacuolation, so that there would evidently have been an ultimate outbreak of frank diabetes on the diet taken except for the untimely death.

TABLE IV.

Dog B2–43.

Date.	Weight, kgm.	Dosage, gm. per kgm.	Administration.	Glycosuria. gm.	Remarks.
1914					
Feb. 4	9.2	5.0	Subcutaneous injection	Faint	Normal dog.
Mar. 26	11.1	5.0	Subcutaneous injection	Faint	
April 3	10.2	6.0	Subcutaneous injection	0	
20	11.6	7.0	Subcutaneous injection	0	
27	10.3	7.0	Subcutaneous injection	0	
May 20	Processus uncinatus stripped of peritoneal covering, vessels ligated.				
June 2	11.0	7.0	Subcutaneous injection	0	
16	Processus uncinatus removed.				
26	9.9	7.0	Subcutaneous injection	Slight	
July 9	Processus lienalis removed.				
30	10.3	1.0	Stomach tube	0	
Nov. 9	..	4.0	Subcutaneous injection	Faint	

REMARKS ON DOG B2–43.

The dog was received in a state of fair nutrition and the original weight of 9.2 kgm. was used as the basis of reckoning dosage throughout. Thus the identical quantity of 46 gm. glucose was injected subcutaneously on February 4 at this weight, and on March 26 after the animal had been fattened to 11.1 kgm. The same trace of glycosuria occurred each time, perhaps from nervousness, for after becoming accustomed to the procedure the dog tolerated 64.4 gm. (7 gm. per kilo) without glycosuria, as she normally should do. The point

here is that the changes of weight, which would be sufficient to affect the tolerance of a diabetic dog to a marked degreé, had no such influence upon the assimilation of the normal animal.

On May 20 the uncinate process of the pancreas was stripped of peritoneum and the vessels and nerves entering its tip divided—in other words the operation of removal was performed except the actual removal, in order to test whether the lowering of apparent tolerance is due to actual removal of tissue or partly to trauma or nervous or circulatory disturbances. After this, 7 gm. of glucose per kilo was still tolerated without glycosuria; but after actual removal of the uncinate process, on June 16, the same dose caused slight glycosuria. Removal of the splenic process on July 9 lowered the tolerance further, so that on November 9 faint glycosuria followed the injection of 4 gm. per kilo.

REMARKS ON DOG B2–60.

This Great Dane was fat when received and 45 kilos was assumed as the normal weight on which to reckon dosage throughout. According to paper 1 of Series I such a large dog would naturally have a small pancreas in proportion to the body weight, and by adding the weights of the portions of pancreas at operations and at autopsy, this particular animal was found to have 1.4 gm. of pancreas per kilo on 45 kilos weight. The tolerance is seen, nevertheless, to be fully as high as that of the normal dogs preceding.

The dog at first did not thrive in confinement and the weight gradually fell from 48.6 to 39.4 kilos. This difference was sufficient to raise the ratio of pancreas weight to body weight appreciably and would have increased the tolerance of a diabetic dog markedly. The complete assimilation of 9 gm. of glucose per kilo by stomach tube on July 13 and the excretion of 0.6 gm. from the same dose subcutaneously on December 13 represented about the average tolerance and indicated no rise of tolerance in the normal animal from diminution of body weight.

After removal of the splenic process of the pancreas on December 17 it will be observed that the tolerance was lowered so that faint glycosuria resulted from as little as 2 gm. of glucose per kilogram subcutaneously and an appreciable excretion from 4 gm. per kilo

TABLE V.

Dog B2–60.

Date.	Weight, kgm.	Dosage, gm. per kgm.	Administration.	Glycosuria, gm.	Remarks.
1914					
May 19	48.6	3.0	Subcutaneous injection	0	Normal dog.
June 2	46.4	5.0	Subcutaneous injection	0	
8	..	6.0	Subcutaneous injection	0	
26	41.8	7.0	Subcutaneous injection	Faint	
July 8	40.0	7.0	Stomach tube	0	
13	39.2	9.0	Stomach tube	0	
Dec. 13	39.4	9.0	Subcutaneous injection	0.6	
17	Removed processus lienalis of pancreas weighing 26.6 gm.				
1915					
June 2	38 0	4 0	Subcutaneous injection	0.52	
11	38.0	2 0	Subcutaneous injection	Faint	
18	36.4	4 0	Stomach tube	0.76	
30	39.2	4.0	Subcutaneous injection	Faint	In the first twenty-four hours after the injection 344 c.c. urine, faint glycosuria; in the second twenty-four hours after injection (fasting), 2537 c.c. urine, sugar-free.
1916					
Feb. 17	45.0	12.0	Stomach tube	0.17	1 hour after feeding.
				1.22	2 hours after feeding.
				1.31	3 hours after feeding.
				0.25	4 hours after feeding.
	.			Faint	5½ hours after feeding.
Mar. 7	Removed pancreas tissue weighing 12.7 gm.				
May 8	45.0	12.0	Stomach tube.	1.72	
16	Removed pancreas tissue weighing 6.81 gm.				
June 5	..	12.0	Stomach tube	1.58	4½ hours after feeding.
				0.52	6½ hours after feeding.
				0	24 hours after feeding.
20	Removed pancreas tissue weighing 9.45 gm.				
July 27	35.5	12.0	Stomach tube	7.58	5½ hours after feeding.
				9.59	20 hours after feeding. 49.1 gm. sugar lost in feces.

either subcutaneously or by stomach tube. Attention may be called incidentally to the fact that in this and numerous other experiments abundant time was allowed to pass to exclude the effects of operative trauma and assure that the alteration of tolerance was permanent.

The diminution instead of increase of diuresis in such tests was sufficiently emphasized in a former publication and therefore the urine volumes are mostly omitted from these records. But it may be repeated that when the tolerance is lowered so that small doses, with minimal osmotic influence, maintain hyperglycemia and slight glycosuria longer than in normal animals, the rule still holds that there is marked oliguria during this period and polyuria afterward, as noticed in connection with the experiment of June 30, 1915. ·

At the same time, as frequently mentioned elsewhere, this marked lowering of tolerance in a non-diabetic animal is apparent and not real, in the sense that it does not represent any limitation of the actual assimilative power. This was shown by the administration of 12 gm. of glucose per kilo on February 17, 1916, when the absolute dose was 540 gm. and the total excretion 3 gm., *i.e.*, only the barest trifle more than a normal dog eliminates from the same dose. Higher dosage would have been the only requisite to obtain still higher assimilation.

Successive portions of pancreas were then removed, at first without any apparent effect upon the assimilation of the 12 gm. dose of glucose. Finally the operation of June 20, 1916, brought the animal close to the verge of diabetes. Active diabetes might in fact have been present except for the low body weight of 35 kilos at. that time, due to impaired digestion and diarrhea. In previous tests the feces had been free from sugar, but on July 27 there was diarrhea, with loss of 49.1 gm. glucose. Nevertheless, the sharp change from the former condition is seen in the excretion of over 17 gm. glucose in the urine.

The dog's general health prevented continuing the tests into later stages of diabetes. The principal point of the experiment was that a large dog, with a small endowment of pancreas in proportion to body weight, possessed the average glucose tolerance of the species, and this tolerance declined no more than usual with the removal of successive portions of pancreas.

REMARKS ON DOG B2–61.

Several experiments were undertaken with much smaller animals than this, but they were not strong enough to go through the repeated tests and operations and resist distemper and other accidents. This animal was a small black mongrel, fat when received at a weight of 5.5 kilos, so that 5 kilos was assumed as the normal weight for reckon-

TABLE VI.

Dog B2–61.

Date.	Glucose dosage, gm. per kgm.	Weight kgm.	Administration.	Glycosuria, gm.	Remarks.
1914					
May 27	8.0	5.5	Subcutaneous injection	0.24	Normal dog.
June 2	6.0	5.5	Subcutaneous injection	Faint	
8	5.0	5.6	Subcutaneous injection	0	
26	7.0	6.0	Subcutaneous injection	0	
July 8	8.0	6.5	Subcutaneous injection	Faint	
13	8.0	6.1	Stomach tube	0.10	
17	9.0	6.4	Subcutaneous injection	0.30	
30	7.0	6.5	Stomach tube	Faint	
Aug. 12	8.0	6.3	Stomach tube.	0	
Nov. 9	8.0	6.0	Subcutaneous injection	0.20	
13	Removed tissue weighing 11.74 gm., about two-thirds of pancreas.				
1915					
June 2	3.0	..	Subcutaneous injection	Slight	3½ hrs. after injection.
				Slight	9 hrs. after injection.
				0	22 hrs. after injection.
11	1.5	6.7	Subcutaneous injection	Faint	5 hrs. after injection.
				0	7 hrs. after injection.
18	3.0	6.9	Stomach tube	0.32	3 hrs. after feeding.
				0	10½ hrs. after feeding.
30	4.0	6.6	Subcutaneous injection	Faint	18 hrs. after injection.

ing purposes throughout. She became more obese under the conditions of cage life. According to general rules a high ratio of pancreas weight to body weight was to be anticipated and the actual finding was that this animal possessed 3.75 gm. of pancreas tissue per kilogram.

The glucose tests in the normal state indicated a slightly lower apparent tolerance both subcutaneously and by stomach tube than in Dog 260.

November 13, 1914, the two processes, representing about two-thirds of the pancreas, were removed, leaving most of the body of the gland in place. The tolerance was lowered so that faint glycosuria resulted from as little as 1.5 gm. of glucose per kilo subcutaneously on June 11, 1915.

Accordingly a small dog with a high ratio of pancreas to body weight has no higher glucose tolerance than a large dog, and there is no greater "margin of safety," as judged by the lowering of the apparent tolerance by removal of pancreatic tissue. It was already concluded from the compilation of records in paper 1 of Series I that there is no uniform difference, outside of the limits of error, in the proportion of pancreatic tissue which must be removed to produce diabetes in large and small dogs.

REMARKS ON TABLE VII.

Instead of single dogs with removal of successive portions of pancreas this table is made up of different dogs possessing different portions of the gland. The "Remarks" column is occupied mostly with the fractions of pancreas remaining. Though the body weights frequently varied, the original weight in each instance is used as the standard for dosage in all tests.

The normal dog No. B2–40 tolerated 10 gm. glucose per kilo subcutaneously when first received. The animal refused all food in the strange surroundings, so that from January 28 to February 3 the weight fell from 11.7 to 10.6 kilos. A smaller glucose injection was then given (6.7 gm. per kilo on the original 11.7 kilo weight), with the result of high glycosuria in a very small urine volume. A distinction must be made between this "hunger glycosuria" and the effect of simple changes of weight. A normal dog is unchanged in tolerance by changes of weight, but is subject to this transitory glycosuria or hyperglycemia on receiving carbohydrate after long fasting or scanty diet. The true tolerance of a diabetic animal rises when the weight is reduced even by fasting, but in mild cases the sudden giving of carbohydrate after fasting or even after protein-fat diet often causes a transitory glycosuria like that of normal animals, but heavier.[4]

TABLE VII.

Dog No.	Date	Weight kgm	Glucose dosage, gm per kgm	Administration	Glycosuria, gm.	Remarks.
B2–40	Jan. 28	11 7	10 0	Subcutaneous injection	Faint	Normal dog.
	Feb. 3	10 6	6 7	Subcutaneous injection	3.8% in 7 c.c. urine	Weight lost by refusal of food.
C3–91	May 8	18 0	12 0	Stomach tube	1 1	Normal dog.
B2–59	19	41.8	3 0	Subcutaneous injection	0	Normal dog.
	June 2	40.0	5 0	Subcutaneous injection	0	
	8	..	6.0	Subcutaneous injection	0	
	26	40.0	7 0	Subcutaneous injection	Faint	
	July 7	39.1	9.0	Stomach tube	0 36	
	13	38.2	7 0	Stomach tube	0 76	
	30	42 5	5 0	Stomach tube	0	
B2–62	June 2	7 7	3 0	Subcutaneous injection	Slight	Only uncinate process present one-third to one-fourth of pancreas.
	8	7.6	2 0	Subcutaneous injection	Faint	
	26	8.3	4 0	Subcutaneous injection	0	
	July 2	..	4 0	Stomach tube	0	
	7	8 3	6.0	Stomach tube	0	
	13	8 4	7.0	Stomach tube	0	
	17	9 2	8 0	Stomach tube	Slight	
	30	9 5	8 0	Stomach tube	Slight	
	Nov. 24	Removed pancreatic tissue weighing 0.2 gm.				
	Mar. 30	..	9 0	Subcutaneous injection	0	
	April 8	..	8 0	Stomach tube	1 25	
B2–44	June 26	22 2	6 0	Subcutaneous injection	Faint	One-fourth to one-fifth of pancreas.
	July 2	..	6 0	Stomach tube	0	
	8	22.8	7 0	Subcutaneous injection	Faint	
	17	26.9	7.0	Stomach tube	0	
	30	21.0	9 0	Stomach tube	0	
B2–38	Feb. 10	12.1	1.0	Stomach tube	Faint	One-sixth of pancreas.
B2–57	July 8	11.3	3.0	Stomach tube	2.9	One-seventh of pancreas.
	13	..	2.0	Stomach tube	2.7	
B2–26	Dec. 30	19.0	2.0	Subcutaneous	0 23	One-ninth of pancreas; non-diabetic.
	Jan. 2	..	1.0	Subcutaneous	0	
	15	..	1.2	Subcutaneous	0.15	

TABLE VII—*Concluded*.

Dog No.	Date.	Weight, kgm.	Glucose, dosage, gm. per kgm.	Administration.	Glycosuria, gm.	Remarks.
B2–71	June 26	13.1	3.0	Subcutaneous	0	One-ninth of pancreas, non-diabetic.
	July 8	13.3	4.0	Subcutaneous	0	
	17	13.3	6.0	Subcutaneous	Faint	
	30	13.5	4.0	Stomach tube	0	
	Aug. 12	13.0	6.0	Stomach tube	Faint	
	Nov. 19	11.9	6.0	Subcutaneous	Faint	
B2–10	Jan. 2	7.0	2.0	Subcutaneous	Faint	One-tenth of pancreas.
	5	..	1.5	Subcutaneous	0.15	Diabetes prevented first by cachexia and later by hypertrophy of pancreas remnant.
	9	..	0.9	Subcutaneous	0	
	April 3	6.0	1.2	Subcutaneous	0	
	20	7.0	1.8	Subcutaneous	Faint	
	27	6.3	1.8	Subcutaneous	Faint	
	July 7	10.7	2.0	Stomach tube	1.0	
B2–31	April 27	9 6	..	Stomach tube	1.50	One-eighth to one-ninth of pancreas, diabetic.
		(11.1)	1.0			
C3–13	June 30	13.5	4.0	Subcutaneous injection	12.7	One-eleventh of pancreas, diabetic.

Dogs C3–91 and B2–59 furnish examples of the normal tolerance in large animals; one 18 and the other above 40 kilos in weight.

Dogs B2–62 and B2–44 were examples of exceptionally high tolerance after removal of a considerable part of the pancreas. The former animal was small and the latter large, so the difference did not pertain to size. The ordinary glycosuria of such animals is so slight that it might easily be stopped by any slight cause, and the question whether the obstacle in these cases may have been a slight renal impermeability was not investigated.

Dog B2–38, possessing one-sixth of the pancreas, showed faint glycosuria from 1 gm. glucose per kilo by stomach tube.

Dog B2–57, possessing one-seventh of the pancreas, excreted 2.9 and 2.7 gm. glucose respectively from 3 and 2 gm. per kilo by stomach tube.

Dog B2–26, non-diabetic with one-ninth of the pancreas, excreted titratable quantities of glucose from 2 gm. and from 1.2 gm. per kilo, but none from 1 gm. per kilo, subcutaneously.

Dog B2–71, also non-diabetic, with one-ninth of the pancreas, showed a contrasting behavior, in that glycosuria was absent with 4 gm. and only faint with 6 gm. per kilo, both subcutaneously and by stomach tube. Attention may be called incidentally to the usual close parallelism between the results of administration subcutaneously and by stomach tube in this and other experiments.

Dog B2–10 had been left with only one-tenth of the pancreas, but was non-diabetic at the time of the tests, because illness and emaciation following operation had allowed time for successful hypertrophy of the pancreas remnant. The apparent tolerance subcutaneously lay between 1.2 and 1.8 gm. per kilo, while 2 gm. per kilo by stomach tube caused an excretion of 1 gm. of glucose.

Dog B2–31 was diabetic, with a remnant between one-eighth and one-ninth of the pancreas, but was long kept aglycosuric by a limited protein-fat diet at a reduced body weight. During this period of latent diabetes the animal, at a weight of 9.6 kilos, was given by stomach tube 11 gm. glucose, which was 1 gm. per kilo on the normal weight of 11 kilos. An excretion of 1.5 gm. glucose resulted.

Dog C3–13, diabetic, with one-eleventh of the pancreas and similarly kept free from glycosuria on protein-fat diet, received 52 gm. glucose, or 4 gm. per kilo, subcutaneously, and excreted 12.7 gm.

The last two animals illustrate diabetic characteristics, in that even in latent diabetes small doses of sugar are likely to cause an appreciable excretion, which increases markedly with the dose. As long as the diabetes is kept under control the animal evidently possesses enough of the internal pancreatic secretion to utilize the greater part of any single dose; but with repetition of doses or any other cause of glycosuria the loss of sugar increases, even to the point where the total quantity administered is eliminated. Examples of this behavior during active diabetes have been previously given,[6] so that they need not be repeated here.

[6] Allen, F. M.: Glycosuria and Diabetes, 1913, Chapter VI.

Any gradual character of the decline of apparent tolerance should not cause confusion as to the sharpness of the point at which the reduction of the true tolerance begins. As previously mentioned, this point may be so sharp that the removal of one-tenth of a gram of pancreatic tissue makes the difference. Before the removal of this tenth the excretion from any dosage of glucose given by stomach or subcutaneously is relatively trivial, and the maintenance of even a slight glycosuria permanently (or continuously through any long period of time) is absolutely impossible. After the removal of the tenth of a gram of tissue not only is glycosuria greater, but it can be maintained indefinitely and tends to increase. This is the condition which has always been known clinically as diabetes.

This discussion is based upon the older tests for glycosuria before the introduction of Benedict's new methods,[7] but the distinction is so vital as a boundary between uninterrupted health and a progressive fatal disease that it seems unlikely to be altered in principle by any improved chemical procedure.

DISCUSSION.

Certain quantitative deductions concerning the internal pancreatic function may be drawn from tests in the three degrees of assimilation which have long been recognized clinically.

1. *Normal Assimilation.*—Here the tolerance corresponds to the average for individuals of the same species by all tests. Two factors are necessarily involved, namely, the supply of pancreatic hormone and the capacity of the body cells for storage, combustion or transformation. As the assimilation can always be overtaxed by sufficiently large doses of sugar the question may be raised as to which of these factors becomes deficient in the normal animal. It is rather surprising to find that the pancreatic element is not present in the superabundance ordinarily supposed, but that the removal of any significant fraction, such as a fourth or a third of the pancreas, causes a well-marked lowering of tolerance in the usual tests. In other words an excess of Langerhans tissue to serve as a mere "factor of safety" is not demonstrable; on the contrary there must be frequent

[7] Benedict, S. R., and Osterberg, E.: Jour. Biol. Chem., 1918, xxxiv, 195.

occasions in ordinary life (such as the eating of a box of candy by a human individual), when the full power of this tissue must be exerted if the assimilation is to be strictly normal. It is therefore conceivable that the overtaxing of the pancreatic function is at least one element when the normal tolerance is exceeded, and that if the functional capacity of the pancreas could be augmented the sugar tolerance might be raised.

2. *Lowered Tolerance.*—This is understood clinically as an abnormal tendency to hyperglycemia or glycosuria from carbohydrate, but short of the point of diabetes. It is to be interpreted as a slowing of the rate of sugar utilization by the cells. There are two conceivable conditions of such slowing. (*a*) With any reduction of pancreatic tissue or function the cells of the body may be chronically undersupplied with the hormone. Their ordinary metabolism may or may not be affected by such deficiency, but it becomes plainly evident under conditions of strain; (*b*) the other alternative requires the assumption that the function of the pancreatic islands is delicately adjusted to the immediate demands of metabolism and that an increased absorption of carbohydrate requires an increased flow of their secretion. Under this conception a reduced mass of pancreatic tissue might be fully adequate for ordinary metabolism, but unable to respond perfectly to carbohydrate excess. The two alternatives are not mutually exclusive and might both be true. The latter may seem *a priori* more plausible, but some evidence favors the former idea, namely, the supply of the immediate needs of carbohydrate metabolism by pancreatic hormone stored in the tissues. This evidence consists in Verzár's[8] observation that the respiratory quotient falls only gradually after total pancreatectomy, and that during the first few hours intravenously injected glucose still raises the quotient; and Hédon's[9] assertion that diabetes comes on more quickly after removal of a subcutaneous pancreatic graft which was barely large enough to keep a dog non-diabetic, than after removal of the entire pancreas of a normal dog. If these statements, especially the latter, could be proved beyond the possibility of errors from

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[8] Verzár, F.: Biochem. Ztschr., 1912, xliv, 201; 1914, lxvi, 75. Verzár and v. Fejer, A.: Ibid., 1913, liii, 140.

[9] Hédon, E.: Arch. internat. de physiol., 1913, xiii, 4.

anesthesia, trauma, differences in nutritive state, etc., they would establish (1) that the pancreatic secretion is stored in the tissues, and (2) that the store of it is greater with a normal pancreas than with a small remnant of pancreas. Evidence for the other alternative will be considered in a subsequent review.

3. *Diabetes.*—This has long been identified clinically by continuous or prolonged glycosuria and hyperglycemia. In the mildest diabetes such continuance may require a maximum ingestion of carbohydrate, but nevertheless there is a sharp distinction from any non-diabetic degree of lowered tolerance, in which such a continuance is impossible. A tendency to regard this classic distinction as fictitious has been manifested by some writers who are disposed theoretically to plot the curve of falling tolerance as a diagonal straight line descending from the fully normal level to total diabetes. Thus an animal which had lost one-tenth of its pancreas would be one-tenth diabetic; one that had lost one-fourth of its pancreas would be one-fourth diabetic; one that had lost nine-tenths of its pancreas would be nine-tenths diabetic, etc. Several facts refute this theoretical conception and establish the strict clinical concept of diabetes. (*a*) From the standpoint of gross anatomy, even though all portions of the pancreas are practically equal in potency, the curve of falling tolerance is not a straight line. The importance of a given mass of tissue increases as the total remaining mass decreases. The point is reached where the removal of 1 or 2 grams has greater effect than the removal of 5 to 10 grams in the first place, and diabetes may finally result from the removal of as little as 0.1 gm. (*b*) There is an important distinction in the microscopic anatomy, for hydropic degeneration of islands never occurs in the non-diabetic state no matter how low the tolerance, while it always occurs in diabetes if the island function is sufficiently overtaxed. There is a narrow border condition in which slight vacuolation of islands may be present together with glycosuria or hyperglycemia on high carbohydrate feeding, and yet the vacuolation and the diabetes both clear up permanently; but this is merely the result of hypertrophy of the remnant or subsidence of inflammation, and therefore is not a real exception to the sharpness of the boundary. This is the basis of the tendency to aggravation which is characteristic of diabetes with excessive diets and the complete

harmlessness of any excess in any state of lowered tolerance short of diabetes. (*c*) A third distinction is found in the so-called "paradoxial law" formerly stated by the writer.[10] The lowering of tolerance produced by removal of the greater portion of the pancreas is apparent rather than real. Only a trivial fraction of even the largest dose of sugar is excreted; the assimilation is merely slowed somewhat and remains actually unlimited. But after removal of about seven-eighths

Normal assimilation.
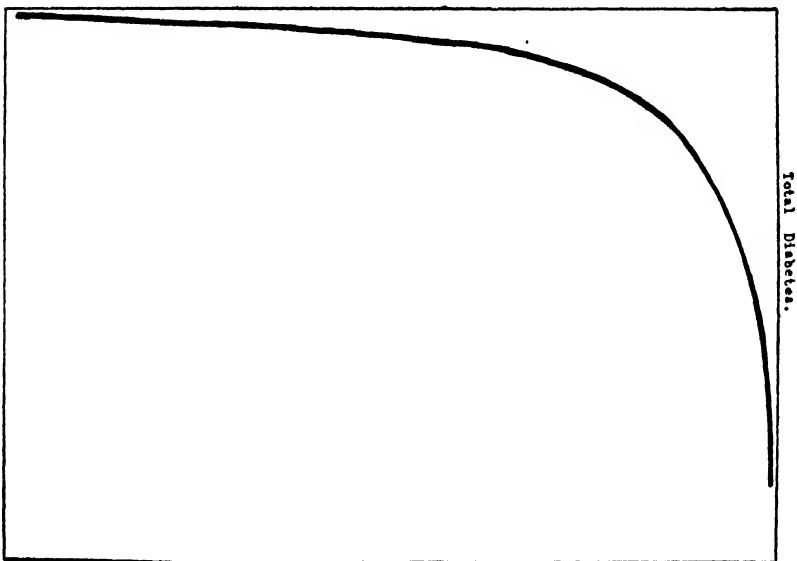
Type of curve of reduction of carbohydrate assimilation by successive removal of portions of pancreas. (Ordinates, fractions of pancreas removed. Abscissæ, carbohydrate tolerance.)

to nine-tenths of the pancreas a difference becomes evident, suddenly or gradually according to the form of the tests. The results of single doses of sugar are not infallible and special circumstances may cause a mildly diabetic animal to show an apparently better assimilation than one that is not quite diabetic. Nevertheless, there is a striking tendency to higher and longer hyperglycemia and glycosuria, which becomes greater the longer the excessive dosage is continued. The

[10] Allen, F. M.: Glycosuria and Diabetes, 1913, p. 67.

actual quantitative excretion is considerable, and with progress to the diabetes or removal of a few small fragments of pancreas it approaches more and more closely to the total quantity administered.

It may be said therefore that the curve of lowered tolerance resulting from the removal of successive portions of pancreas approximates a hyperbola. (See Figure.) Starting at the vertex of this curve one limb may be traced backward as a variable approaching the normal tolerance while the other limb descends as a variable approaching the limit of total diabetes.

CONCLUSIONS.

1. Dogs show an increased tendency to glycosuria from glucose given by stomach or subcutaneously when as little as a fourth or a third of the pancreas is removed. Apparently, therefore, the pancreas has little if any "margin of safety" from the standpoint of strictly normal metabolism, and there may be frequent occasions when its full endocrine function is needed for the purpose of fully normal assimilation. Quantities of sugar which exceed the normal assimilation may possibly be conceived as overtaxing the normal pancreatic function.

2. The internal secretory potency of different parts of the pancreas is equal, as far as such tests can determine; but the influence of a given mass of tissue increases as the total mass of remaining tissue decreases. The "margin of safety" of the pancreas with regard to diabetes is large, amounting in the dog to at least seven-eighths of the gland. The point at which diabetes begins is sharp and definite, according to three criteria: (a) An animal may be brought so close to the verge of diabetes that it is brought on by the removal of as little as 0.1 gm. additional tissue. (b) At this point a new histologic phenomenon begins, namely, the hydropic degeneration of the islands described elsewhere, which is the basis of the characteristic aggravation of diabetes on excessive diets, while such excesses are harmless in any states of lowered tolerance short of diabetes. (c) The lowering of tolerance in any stage short of diabetes is only apparent, representing only a slight delay of assimilation while the actual capacity is unlimited, and the maintenance of continuous glycosuria through any

long period of weeks or months is absolutely impossible by any quantity of sugar or any other food; but in diabetes the limit of assimilation is real and glycosuria progressively increases to the point of total excretion of the quantity administered. The curve of lowering of tolerance, with removal of successive portions of pancreas, is therefore approximately hyperbolic in form. Starting as a variable which descends by successive slight degrees below the level of normal tolerance, it turns at the vertex into a variable which approaches total diabetes as its limit.

3. Certain conceptions concerning the quantitative relations of the pancreatic hormone may be deduced as follows: It stands in some quantitative relation with the amount of carbohydrate metabolized, because a deficiency is revealed by moderate glucose dosage when only one-fourth of the pancreas is removed, and because of the above-mentioned proof that in diabetes the islands can be driven to destructive overfunction by carbohydrate excess and spared by regulation of diet. A more important quantitative relation is the minimum requirement of the body cells to prevent diabetes. When this minimum quantity of the hormone is present the organism retains its power to metabolize almost the whole of any glucose dosage that can be absorbed from the stomach or subcutaneous tissue, no matter how large or how long continued. When this minimum is reduced by only a trifle the phenomena of diabetes begin. With mild diabetes this deficit may be guarded against by restriction of carbohydrate. With more severe diabetes the total diet and body weight must be reduced. With still more severe diabetes the supply of hormone is inadequate for even the lowest metabolism and glycosuria is therefore uncontrollable even by fasting. As an example it may be assumed that a dog becomes diabetic with removal of between seven-eighths and nine-tenths of the pancreas, and in this condition requires maximal starch and sugar feeding to maintain glycosuria: Hopeless diabetes, uncontrollable by fasting, results (barring hypertrophy) when the remnant is about one-twentieth of the pancreas. The absolute difference between these fractions may be, for a fair-sized dog, perhaps 2 grams of pancreas tissue. Accordingly the difference between the demands of the highest possible carbohydrate metabolism and the demands of the lowest possible general metabo-

lism amounts in such an animal to no more than the possible output of 2 grams of pancreatic tissue, only a small fraction of which consists of islands.

Such a calculation is of interest in animals when quantitative estimations can be made with approximate accuracy by operations. There is evidently a fallacy in the application to human patients, for it is impossible that the destruction of islands in human diabetes should always fall within the narrow limits mentioned. As a matter of fact, diabetes uncontrollable by fasting is very common in experimental animals and very rare in human cases. A possible explanation may be that one prominent feature of human cases is a functional defect which interferes with the internal secretory activity of the islands and at the same time renders them specially susceptible to damage from functional overstimulation. Such an explanation is supported by observations in other directions. One of these is the abundance of normal appearing islands in some clinical cases necessitating the assumption of a functional impairment. Another is the wide variation in the susceptibility of different human patients (especially the old and young) to degeneration of islands and corresponding decline of tolerance from dietary excess. At the same time it seems evident that a relatively small mass of normal island tissue can prevent diabetes, and the conclusion is therefore suggested that any positive means of augmenting the endocrine pancreatic function even by a little would give therapeutic results far surpassing those of the negative plan of sparing the function by diet.

EXPERIMENTAL STUDIES IN DIABETES.

SERIES II. THE INTERNAL PANCREATIC FUNCTION IN RELATION TO BODY MASS AND METABOLISM.

2. CHANGES IN ASSIMILATION BY ALTERATIONS OF BODY MASS.

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The preceding paper[1] was a study of the alterations of assimilation produced by changing the mass of the pancreas. The present one deals with the alterations of assimilation produced by changing the body mass. Attention was first given to these relations in obese dogs.

I. PANCREAS AND BODY MASS RELATIONS IN OBESE DOGS.

The following conclusions were drawn (Table I):

1. No anatomic differences were found between different ages or different types of obesity. At one extreme are young animals which are fat apparently for the sole reason that they eat enormously and are very lazy. At the other extreme is a type well known to those who handle many dogs, namely, animals in advanced senility, toothless or with teeth worn to the gums, excessively obese and sluggish, generally either surly or stupid, and consuming sometimes a large and sometimes a surprisingly small quantity of food. Both types of animals in the above series were free from either gross or microscopic changes in the pancreas; in particular, from either fibrous or fatty invasion or island changes. If it is permissible to apply these observations to human pathology, it may be concluded that such changes in the pancreas are a distinct abnormality and not naturally attendant upon either obesity or senility.

2. Even with the extreme degrees of obesity here represented, the ratios of pancreas weight to body weight fell within the normal limits

[1] Allen, F. M.: AM. JOUR. MED. SC., 1920, clx, 781.

TABLE I.

Pancreas and Body Mass Relations in Obese Dogs.

No.	Body weight, kgm.	Weight, total pancreas, gm.	Grams of pancreas per kgm body weight.	Weight of remnant, gm.	Size of fraction.	Diabetes.	Remarks.
1	6.5	17.6	2.71	1.50	$\frac{1}{11}-\frac{1}{12}$	....	Senile, excessively obese; cachexia without glycosuria.
2	7.0	14.7	2.10				
3	7.2	12.2	1.69	1.30			
4	8.9	19.3	2.17	...	...	....	Age three years.
5	9 3	22.8	2.45	2.90	...	....	Senile.
6	9.7	22.0	2.27	...	...	....	Old.
7	12.0	26.3	2.19	3.40	$\frac{1}{8}$	Mild with removal of 0.85 gm. additional in 3 subsequent operations	Obese young dog.
8	14.3	30.8	2.15	3.25	...	....	Moderately old and fat.
9	14.8	31.3	2.11				
10	14.9	32.5	2.18	1.65	$\frac{1}{20}$	Gravis	Age four years.
11	16.4	35.2	2.42	5.60	$\frac{1}{8}-\frac{1}{4}$	....	Not excessively obese.
12	17.0	31.6	1.86	4.60	$\frac{1}{7}$	Levis	Age three years.
13	18.6	22.9	1.61	4.25	$\frac{1}{5}-\frac{1}{6}$	Levis	
14	18.6	39.2	2.11	...	...	....	Age six years.
15	19.0	36.3	1.86				
16	19.2	31.0	1.61				
17	19.5	36.3	1.81	1.20	$\frac{1}{30}$	Gravis	
18	20.0	28.4	1.42	5.50	$\frac{1}{5}$	....	Death in three days.
19	20.5	41.0	2.00	12.00	$\frac{1}{3}-\frac{1}{4}$	....	Age four years; normal wt. estimated at 13 kilos.
20	20.9	37.7	1.80	2.90	...	....	Old.
21	22 8	33.4	1.46	1.30	$\frac{1}{26}$	....	No glycosuria; death within three days.
22	22.8	41.1	1.80	7.50	$\frac{1}{5}-\frac{1}{6}$	None	Age six or seven years.
23	24 3	42.9	1.76	6.10	...	....	"Rather old."
24	25.0	51.2	2.04	6.50	$\frac{1}{8}$	Gravis	Fat but not pathologically obese; glycosuria in spite of prostration.
25	28.5	46.3	1.62	...	...	....	Moderately obese.
26	32.4	61.3	1.89	12.4	$\frac{1}{8}$	Transitory with prolonged glucose diet.	

shown in Table II, Paper 1 of Series I.[2] This is not because this ratio does not change when an animal becomes fat, but merely because the variations in the ratio in different normal dogs are so wide. It is therefore not possible to demonstrate an abnormal susceptibility to diabetes in obesity on the basis of a reduced mass of pancreas in proportion to the body mass.

3. It was mentioned in the preceding paper that alterations of body weight, apart from starvation or cachexia, do not change the glucose tolerance of normal dogs. Similar tests upon obese animals have shown no departure from the normal assimilation, as far as interpretation was possible. Protocols are omitted because there can be no claim to accuracy in comparisons, since reckoning dosage on the obese weight is obviously unfair and only guesses could be made of the normal weight. Nevertheless any serious reduction of tolerance would have been evident, and the conclusion is justified that neither obesity nor senility *per se* involves any demonstrable lowering of glucose assimilation.

4. The types of obese dogs mentioned react differently to partial pancreatectomy. The younger ones generally withstand the operation well. The senile ones very commonly die within one to three days, presumably of the pancreatic intoxication which is called "fat necrosis" from one of its minor accompaniments. The deaths are not due to ordinary shock, because such animals safely undergo control operations involving more trauma and exposure of viscera and sometimes survive total, longer than partial pancreatectomy. Senility is not always a factor, because a few younger dogs with a type of obesity apparently due to endocrine disorder likewise die abruptly after such operations, while some of the old obese kind which retain greater liveliness survive. Injections of pancreas extract have not conferred immunity, but it is sometimes possible to immunize the animals by successive operations, removing only a very small piece of pancreas the first time. The results are not very satisfactory, because the dogs generally either lose their obesity or die before the series of operations is complete.

5. The main question under consideration was whether obese dogs would for any reason be more susceptible to diabetes than normal

2 Allen, F. M.: Jour. Exp. Med., 1920, xxxi, 366.

dogs.   The results with dogs Nos. 12, 13, 24 and 26 in Table I suggest
that this may be the case.   Diabetes was not produced in any of these
animals with larger pancreas remnants than in some of the normal
dogs of Paper 1, Series I, but the proportion of positive to negative
results with remnants of this size is much higher in the obese series.
This latter series is, however, so short that no positive conclusion is
warranted.

II. ALTERATIONS OF BODY WEIGHT IN THE SAME ANIMAL.

It was concluded in Series I that various influences can be most
accurately judged by applying them to partially depancreatized animals
and observing the individual alterations of assimilation.   One impor-
tant procedure would be to depancreatize a dog just short of diabetes
and then determine whether the most extreme fattening could actually
bring on diabetes, *i.e.*, whether obesity can ever be a genuine cause
of diabetes to an extent equal to the removal of a small fraction of a
gram of pancreatic tissue.   This point was not covered by this investi-
gation because of the practical difficulties.   The method actually
used consisted in taking diabetic dogs free from symptoms on fixed
diets and studying the changes of assimilation produced by alterations
of the nutritive level.   Changes of tolerance in inverse relation to
the body weight were mentioned incidentally in Series I, without
strict proof of the causal character of the relation.   The present
experiments were designed to furnish such proof, on different diets,
but particularly including changes of weight produced entirely by
changes of the non-sugar-forming element of the diet, namely, fat.
The difficulties of carrying through such long experiments successfully
were referred to in Series I.

DOG B2–10.

Female, mongrel, white, aged nine months; good condition; weight,
7 kilos.

November 18, 1913, removal of pancreatic tissue weighing 16 gm.
Remnant about main duct estimated at 1.8 gm. ($\frac{1}{10}$).   There was
glycosuria as high as 4 per cent., which with onset of distemper
diminished in spite of bread and glucose feeding and disappeared

December 1. Weight was rapidly lost through anorexia and diarrhea. The lowest level reached was 4.4 kilos. With gradual recovery from the distemper, weight was gained on plain bread and soup diet, and glycosuria ensued when the level of 5.1 kilos was attained on February 19. The diet was then changed to beef-lung *ad libitum*, glycosuria remained absent, and in subcutaneous tolerance tests in March and April there were traces of glycosuria with 2 gm. glucose per kilo (reckoned on the original 7 kilos weight), but none with 1.8 gm. or less. The weight, which was slowly increasing, was 6 to 6.3 kilos during this time. By July 29 the dog was obese at a weight of 10.4 kilos, and on this date glycosuria appeared and persisted during the following week until checked by fasting and reduced diet.

DOG c3–20.

Male, mongrel, brown, aged six to eight years; fair nutrition; weight, 24 kilos.

June 24, 1915, four-fifths of the pancreas was removed and additional tissue on July 16 and 30. Glycosuria was present on glucose and bread feeding, but ceased, and on plain bread diet on August 15 the plasma sugar rose only to 0.143 per cent. and was 0.088 per cent. the following morning. August 16 an additional 0.6 gm. of pancreatic tissue was removed and the diet was changed to beef-lung *ad libitum*. The weight at this time had fallen to 21.3 kgm. On August 25 the plasma sugar during digestion rose to 0.154 per cent., *i.e.*, higher on protein diet than on carbohydrate prior to the last operation. On September 29, at a body weight of 24.8 kilos, the plasma sugar was 0.077 per cent. immediately before feeding, 0.078 per cent. immediately after feeding and 0.161 per cent. five hours later. October 23, at a body weight of 25.8 kilos, glycosuria of 1.1 per cent. suddenly appeared and ceased only with two days of fasting. The former diet was then resumed and on November 1 sugar suddenly reappeared in the urine in 2.4 per cent. concentration.

DOG b2–52.

Female, bull terrier mongrel, brindle, aged five or six years; excellent condition; weight, 12.8 kilos. April 24, 1914, seven-eighths of the pancreas was removed, and glycosuria proving transitory, half the rem-

nant was removed on May 18.   The weight meanwhile had fallen
to 9.3 kgm.   Glycosuria was immediately checked by fasting and a
diet carefully built up, beginning with 50 gm. beef-lung and 50 gm.
suet, and increasing so that by June 15 the dog was eating 1000 to 1200
gm. lung without glycosuria.   July 30, glycosuria began with 0.53
per cent. sugar in 507 c.c. urine, followed by 1.6 per cent. in 836 c.c.
the next day, at a body weight of 11.4 kgm.   Continuous fasting to
August 12 then reduced the weight to 9.3 kgm. as before, and the
diet was built up as before.   The dog would no longer eat the full
quantity of lung, and it was necessary to introduce 50 to 100 gm.
lard for fattening.   On the lard and quantities of lung below 1000 gm.,
glycosuria remained absent until November 25.   Then at a body
weight of 12.5 kilos glycosuria of 0.4 to 2 per cent. was present for
four days, ceased with a single fast-day, remained absent on the
regular diet for a week, then on the same diet was present for two
weeks, the weight during this time ranging as high as 14.3 kgm. and
the animal appearing in splendid condition.   It was then found impos-
sible to stop the glycosuria, and after two weeks of fasting the dog
died of acidosis.

<center>DOG B2–57.</center>

Female, mongrel, yellow, aged five years; good condition; weight,
11 kilos.   Fasting from May 4 to May 21, 1914, reduced the weight
to 8.7 kilos.   On the latter date 19.6 gm. of pancreatic tissue was
removed, leaving a remnant estimated at 2 gm. ($\frac{1}{11}$).   Glycosuria
remained absent while the diet was gradually built up.

June 14, at a body weight of 7.5 kgm., a diet of 1200 gm. beef-lung
was begun, and the dog ate this quantity daily without glycosuria
until July 30.   On this date there was 0.38 per cent. glucose in 390
c.c. urine and the next day 1.7 per cent. in 635 c.c., the body weight
being 10.4 kilos.

Fasting was then continued to August 12, reducing the weight to
8.1 kilos.   The diet was then built up gradually to 500 gm. lung on
August 22, the weight being 7.7 kgm. on that date.   The dog remained
without glycosuria on this diet to September 14, when the weight had
declined to 7.4 kilos.

September 14, 50 gm. lard was added to this diet, increased on
October 5 to 100 gm.   October 20, at a weight of 10.8 kgm., lard

was stopped because the dog was tired of it, and the lung was increased to 1000 gm. daily. The dog was on this diet to November 13 and gained weight to 11.4 kilos.

November 13, 50 gm. lard was added, increased on November 18 to 100 gm. The lard was now eaten and considerable of the lung was left uneaten. December 24 the weight had risen to 16.3 kilos, which was marked obesity. Glycosuria then began and continued (0.3 to 4.2 per cent.) on the same diet to January 4.

Fasting then stopped the glycosuria in two days, but was continued five days, reducing the weight to 14.25 kilos. Lung feeding was then begun cautiously, with 100 gm. the first day to 500 gm. on January 14, when glycosuria of 0.18 per cent. appeared in 186 c.c. urine. This increased to 1.4 per cent. in 240 c.c. urine and ceased with a single fast day, but successive attempts at feeding now caused glycosuria more easily, until on January 31 it resulted from only 150 gm. lung. Fasting then proved unable to stop the glycosuria, and the dog died March 1, still showing 0.9 per cent. sugar in 1295 c.c. urine, with a body weight of 6.8 kilos.

DOG B2–79.

Male, bull terrier mongrel, brindle, aged four or five years; medium nutrition; weight, 15 kilos. November 10, 1914, removal of pancreatic tissue weighing 32.2 gm. Remnant about main duct estimated at 4.7 gm. ($\frac{1}{8}$).

Glycosuria following operation on bread diet ceased on a diet of 800 gm. beef-lung. From February 16 to April 2, 1915, the diet was 1000 gm. of lung, but the dog was cachectic and lost weight down to 11.2 kilos. The diet was then changed to bread and soup *ad libitum*, with benefit to the general health, and glycosuria remained absent to April 7, when the diet of 1 kilo of lung was resumed. The dog was anemic at this time, with a red cell count of 2,700,000, but began then to improve in digestion and health, gradually regained the original weight and vigor and was used for exercise and other incidental experiments, which did not lower the tolerance. The exercise in fact seemed to prevent glycosuria, as described in the following paper.

In September glycosuria could no longer be restrained on the diet of 1 kilo of lung but was absent on a diet of 500 gm. lung and 500 gm. suet.   The dog was greedy for the suet, and by October 3 was obese at a weight of 17 kilos.   Glycosuria of 0.77 to 1.5 per cent. was then present October 3 to 6.

October 7 to November 8 the dog was fed nothing but suet or lard. At first these were eaten abundantly, but at the end both appetite and digestion were spoiled.   The weight diminished to 13.5 kilos. Glycosuria ceased the first day and blood sugar analyses reported elsewhere showed that the fat had no tendency to create hyperglycemia.   Nevertheless, when a diet of 500 gm. lung was begun on November 9, glycosuria (0.84 per cent. in 323 c.c.) appeared on November 11, *i. e.*, there was not the rise of tolerance which occurs with such a fall of weight under other conditions.

Glycosuria was then kept absent with reduction of protein, while the weight was kept slightly below and above 14 kilos by the use of as much fat as the dog would take.   Beginning December 7, the diet was 300 gm. lung and 200 gm. suet.   Glycosuria was absent until January 2.

January 2, 1916, there was glycosuria of 1.5 per cent. in 194 c.c. urine, ceasing with a single fast day.   Again, the protein was diminished, making the diet 200 gm. lung and 200 gm. suet.   The weight was 14.2 kilos.   It was possible to increase the suet to 300 gm. and then to 400 gm., so that by January 30 the dog weighed 14.9 kilos. Glycosuria then returned.

DOG B2–80.

The earlier history of this animal was given in Paper 2 of Series I.[3]   The carbohydrate diet and glycosuria in April and early May, 1915, were carried to such a point that the dog could not tolerate 1000 gm. of lung at a weight of 15.5 kilos.   Repeated trials of this diet, interspersed with fast days, reduced the weight by June 2 to 13.2 kilos.   The kilo of lung was then tolerated until June 9, when there was 0.17 per cent. sugar in 681 c.c. urine, followed by 0.72 per cent. in 607 c.c. the next day.

[3] Allen, F. M.: Jour. Exp. Med., 1920, xxxi, p. 383.

The glycosuria ceased with a single fast day and the diet was changed to 500 gm. lung and 300 gm. suet. Weight was gained very rapidly and on June 21 there was a return of glycosuria at a weight of 17 kilos.

This glycosuria required two days of fasting to stop and the protein was then further reduced, making the diet 250 gm. lung and 300 gm. suet. The weight continued to increase and the dog had the appearance of splendid health. The photograph at the end of Series I[4] was taken July 2, at a weight of 18.2 kilos. On July 10 there was a sudden appearance of 1.4 per cent. sugar in 605 c.c. urine.

After two fast days to stop the glycosuria the diet of 250 gm. lung and 300 gm. suet was resumed and the dog was used for exercise experiments, as described in the following paper. Increasing glycosuria and acidosis led to death in coma on August 14.

DOG B2–01.

Female, large fox-terrier mongrel, aged two years; good condition; weight, 14 kilos. This dog was received October 25, 1913. Successive removals of pancreatic tissue were carried out and tests of the tolerance were made as described in the preceding paper. Diabetes was finally produced by the removal of 0.8 gm. pancreatic tissue on August 31, 1916. The following record contains some details already referred to in the preceding paper and in Series I.[5]

SUMMARY.

Beginning September 1, 1916, at a weight of 11.4 kgm., the dog was free from glycosuria not only on unlimited bread diet but even with addition of 300 gm. glucose. With continuance of plain bread feeding and a slight gain of weight to 11.7 kgm. the tolerance by November 15 had fallen so that a considerable percentage of glycosuria resulted from giving 56 gm. glucose by stomach tube. The fall in tolerance was further indicated by the repeated glycosuria on bread diet up to December 25; sugar would appear and be checked by fasting; a few days later it would reappear and be checked again; and

[4] Ibid., Fig. 7, Plate 66, following page 608.
[5] Allen, F. M.: Jour. Exper. Med., 1920, pp. 396, 564, 570, 600.

notwithstanding the slight loss of weight, there was the usual gradual decline of tolerance on this program, as indicated by the two days of fasting necessary, December 26 to 27, in contrast to the single days which previously sufficed.

From December 28, 1916, the only food given was a quantity of bread and soup mixture measured in a cup so as to be roughly uniform though not weighed, and representing a slight undernutrition diet, so that there was a gradual fall in weight to 9 kgm. on February 22, 1917. The tables show that at this low weight the blood sugar was normal both before feeding and during digestion of this diet, and the assimilation as judged by a test-meal on February 23 was notably higher than at the higher weight in the preceding November.

Beginning February 24, 100 gm. beef-lung and 100 gm. suet were given daily in addition to the above cup of bread and soup, so as to fatten the dog. The weight thus rose to 14.5 kgm. on June 8 and to 15.25 kgm. on June 12. Glycosuria occurred on June 8, was absent the next day and was continuous June 10 to 12.

Beginning June 13 the diet consisted of 400 gm. lung and 200 gm. suet without carbohydrate. The weight rose to a maximum of 16.6 kgm., and the standard test-meal on June 26 showed a marked fall in assimilation. There was no other glycosuria, but evidently continuous hyperglycemia, as judged by the plasma sugar of 0.147 per cent. before feeding June 30.

Beginning July 3 the diet was only 450 gm. lung, in order to give low calories with as much protein as in former diets. The weight thus fell gradually to 8 kgm. on December 20. On November 15, the addition of 50 gm. bread caused transitory glycosuria, as frequently results from carbohydrate after a carbohydrate-free diet; but there was none with 100 gm. bread the next day and only a slight reaction with 200 gm. on November 17. Rising assimilation was also indicated by the feeding tests of October 5, November 22 and December 18 (Table III). The hypoglycemia present on December 2 (and presumably the following days) corresponded to the emaciation and cachexia.

Beginning December 21 the diets were such as to produce gain in weight. Up to December 31, bread in quantities similar to those given a year previously was tolerated without glycosuria but with

TABLE II.

Dog B2–01.

Date.	Weight, kgm.	Diet.	Remarks.
1916. Sept. 1–4	11.4	Bread and soup *ad libitum*	No glycosuria.
5	11.5	Same, with 100 gm. glucose	No glycosuria.
6	11.5	Same, with 200 gm. glucose	No glycosuria.
7 8–Nov.	11.5	Same, with 300 gm. glucose	No glycosuria.
14	11.5–11.7	Bread and soup *ad libitum*	No glycosuria.
Nov. 15	11.7	56 gm. Merck glucose in 30 per cent. solution by stomach tube (4 gm. per kgm. on 14 kgm. weight; no other food)	Before feeding, sugar neg.; two hours after, 18 c.c. urine, glucose 6.28 per cent.; five and one-half hours after, 38 c.c urine, glucose 4.64 per cent.
16–30	11.6–11.1	Bread and soup *ad libitum*	No glycosuria except with tests (see below).
Dec. 1	11.1	Bread and soup *ad libitum*	1.5 per cent. sugar in 743 c.c. urine.
2	11.1	Bread and soup *ad libitum*	1.85 per cent. sugar in 581 c.c. urine.
3	.....	Fasting	No glycosuria.
4–9	11.0	Bread and soup *ad libitum*	No glycosuria.
10	11.0	Bread and soup *ad libitum*	0.65 per cent. sugar in 1025 c.c. urine.
11	11.0	250 gm. beef-lung	No glycosuria.
12–16	.....	Bread and soup and 100 gm. lung	No glycosuria.
17	.....	Bread and soup and 100 gm. lung	Slight glycosuria.
18–19	10.9	Bones only	No glycosuria.
20–24	.....	Bread and soup and 100 gm. lung	Slight glycosuria
25	11.2	Bread and soup and 100 gm. lung	0.6 per cent. sugar in 260 c.c. urine.
26–27	.....	Fasting	Glycosuria ceased December 27.
Dec. 28, 1916– Feb. 22, 1917	Gradual fall to 9	Small cup of bread and soup daily	No glycosuria except with tests (see below); on February 19, plasma sugar before feeding 0.091 per cent. six hours after 0.133 per cent.

TABLE II—*Concluded.*

Date.	Weight, kgm.	Diet.	Remarks.
Feb. 24–June 7	Gradual rise to 14.5	Same cup of bread and •soup, with 100 gm. lung and 150 gm. suet	No glycosuria except with tests (see below); on February 24, plasma sugar before feeding 0.102 per cent.
June 8	14.5	Same	0.9 per cent. sugar in 105 c.c. urine.
9	.....	Same	No glycosuria.
10	.....	Same	0.5 per cent. sugar in 160 c.c. urine.
11	.....	Same	Slight glycosuria.
12	15.25	Same	0 4 per cent. sugar in 200 c.c. urine.
13–July 2	Gradual rise to 16.6	400 gm. lung and 200 gm. suet; no bread	No glycosuria except with tests (see below); on June 30, plasma sugar before feeding 0.147 per cent.
July 3–Nov. 14	Gradual fall to 9.6	450 gm. lung only	No glycosuria except with tests (see below).
Nov. 15	9.6	450 gm. lung and 50 gm. bread	Faint glycosuria.
16	.....	450 gm. lung and 100 gm. bread	No glycosuria.
17	.....	450 gm. lung and 200 gm. bread	Slight glycosuria.
18–Dec. 20	Gradual fall to 8	450 gm. lung only	No glycosuria except with tests (see below); on December 2, plasma sugar before feeding 0.044 per cent., four hours after 0.110 per cent.
Dec. 21–30	Gradual rise to 8.75	Lung and suet *ad libitum,* with small cup of bread and soup	No glycosuria; on December 27 plasma sugar before feeding 0.145 per cent., four hours after 0.164 per cent., seven hours after 0.147 per cent.
Dec. 31, 1917–Feb. 13, 1918	Gradual rise to 9.2	400 gm. lung and 50 gm. suet	No glycosuria; on January 15, plasma sugar before feeding 0.110 per cent., six hours after 0.130 per cent.; on February 4, plasma sugar before feeding 0.089 per cent.
After Feb. 13, 1918	Gradual rise	400 gm. lung and 100 gm. suet	No glycosuria up to August, 1918.

hyperglycemia. Bread was then omitted and the weight steadily rose on protein and fat.

Events prevented further accurate tests, but glycosuria was absent up to August, 1918, and the animal was fat at that time. Paper 5 of Series I[6] gave the subsequent history up to death on October 15, 1919.

<div align="center">REMARKS.</div>

1. The first four tolerance tests (Table III) were arranged for judging the influence of the tests themselves. The quantity of 30 gm. glucose on November 20, 1916, was guessed at random, and proved smaller than desirable for a standard, as the glycosuria and hyperglycemia were too slight. A diet with 75 gm. glucose was given the next day and again on November 23. The test with 30 gm. glucose was repeated on November 27. As these closely spaced tests showed no significant differences between November 20 and 27 and November 21 and 23 it was concluded that the much less frequent subsequent tests could not be held responsible for any great changes of tolerance. This expectation was confirmed by the later observations of upward as well as downward fluctuations of the assimilation.

2. Attention may again be called to the close reproduction of typical human histories in the record of such an animal. Starting with an excellent physical condition and a very high tolerance it may be said that the occasional tolerance tests correspond to a few violations of diet, but that otherwise almost continuous freedom from glycosuria was maintained; yet the course was downward to death from diabetes three years later. Such a clinical record might well be adduced as evidence of inherent and inevitable downward progress, yet the dietary causes in this animal are perfectly plain; and if the previous conclusion that such dogs are free from inherently progressive tendencies be well founded, it is evident that the diet was the sole cause in this instance.

3. This experiment was planned to include the question of the specific relation of carbohydrate to the pancreatic function, which was also raised in the experiment on Dog 428 in Paper 4 of Series I.[7] The

[6] Jour. Exper. Med., 1920, xxxi, 600.
[7] Jour. Exper. Med., 1920, xxxi, 581–586.

TABLE III.

Feeding Tests.

Dog B2–01.

Date,	Weight, kgm.	Plasma sugar. per cent.	Urine.			Remarks.
			Vol , c.c.	Glucose, per cent.	Total glucose, gm.	
Nov. 20, 1916	11.6	. . .	. . . .	. . . .	0.13	Fed 200 gm. bread, 100
Before feeding	. . . .	0 088	626	Neg.	. . . .	gm. lung and 30 gm.
2 hours after	. . . .	0.133	25	Very faint	. . . .	glucose.
4 hours after	. . . .	0.143	26	0 31	. . . .	
6 hours after	. . . .	0 164	18	0 27	. . . .	
Nov. 21, 1916	11.6	0.094	230	Neg.	0 42	Fed 200 gm. bread, 100
2 hours after	. . . .	0.224	18	Faint	. . . .	gm. lung and 75 gm.
4 hours after	. . . .	0 256	30	0 68	. . . .	glucose.
6 hours after	. . . .	0 159	26	0 83	. . . .	
Nov. 23, 1916	11.6	0 103	110	Neg.	0 33	Same diet as Nov. 21.
2 hours after	. . . .	0 147	22	0.46	. . . .	
4 hours after	. . . .	0.182	22	0.38	. . . .	
6 hours after	. . . .	0.200	32	0 48	. . . .	
Nov. 27, 1916	11.6	0 093	20	Neg.	0.20	Same diet as Nov. 20
2 hours after	. . . .	0.130	78	Very faint	. . . .	
4 hours after	. . . .	0 135	48	0 42	. . . .	
6 hours after	. . . .	0.095	27	Faint	. . . .	
Feb. 23, 1917	9 0	0 135	46	Neg.	0 22	Same diet as Nov. 21 .
2 hours after	. . . .	0.128	17	0 42	. . . .	.
4 hours after	. . . .	0.154	15	0 43	. . . .	
6 hours after	. . . .	0.178	21	0 43	. . . .	
May 17	13 5	0 078	75	Neg.	4.79	Same diet as Nov. 21.
2 hours after	. . . .	0.212	16	2.14	. . . .	
4 hours after	. . . .	0.242	34	5 18	. . . .	-
6 hours after	. . . .	0 250	45	6 42	. . . .	
June 26	16 6	0.132	7	Neg.	10 35	Same diet as Nov. 21.
2 hours after	. . . .	0.345	32	2.99	. . . .	
4 hours after	. . . .	0.416	92	4.33	. . . .	
6 hours after	. . . .	0 370	110	4.93	. . . .	
Oct. 5	11.5	0.133	21	Faint	1.69	Same diet as Nov. 21.
2 hours after	. . . .	0.263	12	4.32	. . . .	
4 hours after	. . . .	0.250	15	4.23	. . . .	
6 hours after	. . . .	0.220	17	3.20	. . . .	
Nov. 22	9.8	0.123	64	Neg.	4.85	Same diet as Nov. 21.
2 hours after	. . . .	0.303	44	4.20	. . . .	
4 hours after	. . . .	0.256	27	5.61	. . . .	
6 hours after	. . . .	0.227	30	4.94	. . . .	
Dec. 18	8.0	0.147	9	Neg.	0.21	Same diet as Nov. 21.
2 hours after	. . . .	0.141	7	Faint	. . . .	
4 hours after	. . . .	0.175	11	0.78	. . . .	
6 hours after	. . . .	0.161	7	1.66	. . . .	

initial high carbohydrate diets undoubtedly reduced the assimilation more rapidly and powerfully than any other food, and at all stages carbohydrate obviously created the greatest tendency to glycosuria. On the other hand this experiment demonstrated (still more strongly than that on Dog 428), in the period December 28, 1916, to February 22, 1917, that with under-nutrition a diabetic animal may live on chiefly carbohydrate diet without glycosuria and with normal blood sugar. In addition to refuting any exaggerated ideas concerning carbohydrate as a sole and specific poison in diabetes, this serves to check too great simplicity in the interpretation of the action of dietary restriction. To some extent it is doubtless true that the food carbohydrate and the body glycogen give rise to glycosuria, and it might be assumed that fasting and restricted diet merely reduce these two sources of urinary glucose. But it is a safe assumption that an animal undergoing slight gradual undernutrition on a bread diet has more glycogen than on a diet of 400 gm. beef-lung and 200 gm. suet (especially in view of the known influence of fat feeding in diminishing glycogen). The experiment then shows that the blood sugar may be lower and the tolerance higher under the former than under the latter conditions. In other words, diabetic glycosuria must be governed by something deeper than merely the quantity of carbohydrate available either in the food or in glycogen.

4. Attention was also paid to the protein ration, which was kept nearly enough equal so that fluctuations in the tolerance could not be attributed to it. Presumably the sugar which caused the hyperglycemia of June 30, 1917, and also that excreted in the active diabetes of 1919, was derived from protein, but the assimilation of this protein obviously varied widely with changes in body weight.

5. The changes in body weight were due essentially to fat feeding and fat deposit, and an influence of fat in connection with sugar metabolism and diabetes is thus demonstrated. The question whether the relation to the internal pancreatic function is direct or indirect is not answered by this experiment. On one hand is the predominant effect of carbohydrate in breaking down assimilation, as already mentioned; in the initial mild stage it is doubtful if the tolerance could ever have been broken down by fat or any non-carbohydrate diet. On the other hand, even in the later stage, carbohydrate was apparently
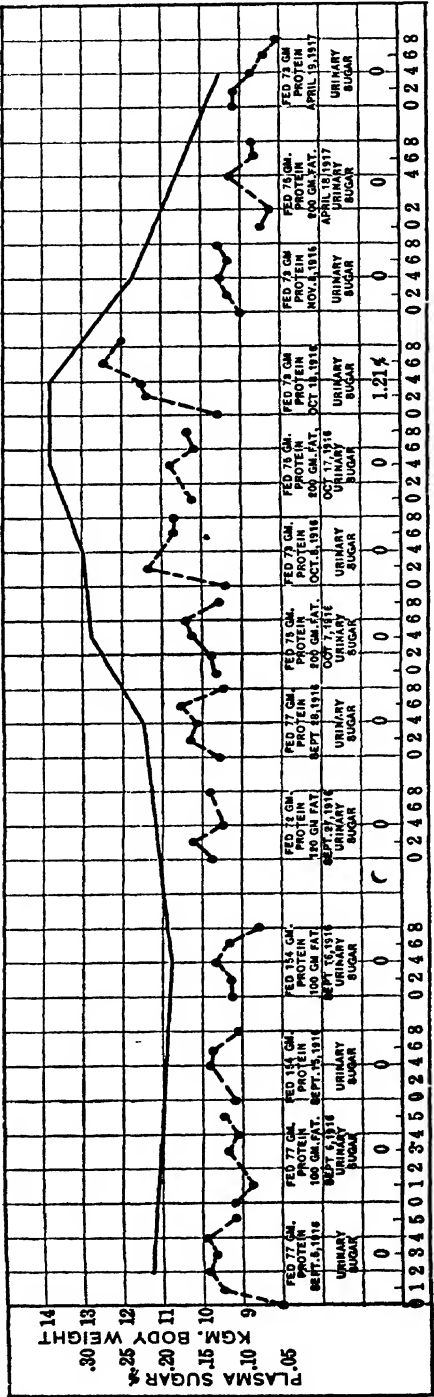
CHART OF TESTS PERFORMED ON DOG C3-86

assimilated well and harmlessly at a low level of nutrition, while over-
nutrition gradually brought on diabetes even in absence of preformed
carbohydrate. In either case the overload of assimilation was mani-
fested by glycosuria or hyperglycemia. There was no evidence of
harm from either carbohydrate or gain of weight so long as these signs
of overstrain of the carbohydrate side of metabolism were avoided;
but forcing either the carbohydrate ration or the total nutrition beyond
the limits set by these danger signals resulted in downward progress
and finally hopeless diabetes.

<div align="center">DOG C3–86.</div>

Female, mongrel, brown, aged two or three years, slightly obese;
weight 15 kilos. April 28, 1916, removal of pancreatic tissue weigh-
ing 33 gm. Remnant about main duct estimated at 2.9 gm. ($\frac{1}{12}$ to
$\frac{1}{13}$). Glycosuria was checked by fasting and undernutrition and the
dog was kept in this border-line condition under careful tests until it
was established that the assimilation limit amounted to about 800
gm. of beef-lung and that this tolerance showed no further tendency
to change spontaneously. Beginning September 5 a series of tests
were performed, most of which were previously published,[8] and the two
concluding ones are now added in the accompanying graphic chart.

The points of chief importance in this experiment are the following:
(1) The protein of the diet was kept constant throughout and the
changes of weight were produced solely by adding and withdrawing
fat; (2) there was no indication of the conversion of fat into sugar—
on the contrary, the plasma sugar curves were generally distinctly
lower on the test days with protein plus fat than on the days with
protein alone, probably because of slower absorption with the fat
admixture; (3) under these conditions it was proved decisively that
the assimilative power fell as the weight rose, and was restored even
higher than the original level when the body weight was reduced
below the original level.

<div align="center">III. ALTERATIONS IN BODY MASS IN THE FORM OF ACTIVE
PROTOPLASM.</div>

In the preceding paper have been described the phenomena re-
sulting from reduction of the mass of the pancreas and in the present

[8] Allen, F. M.: AM. JOUR. MED. SC., 1917, p. 153, Chart VIII.

paper have been discussed the changes in metabolism resulting from the reduction of body mass in the form of reserves, especially fat, by alteration in diet.   It now remains to discuss the changes resulting when the body mass is suddenly reduced through loss of body tissue, such as may occur following extensive injury, or following amputation.   The latter is unfortunately frequently necessary in diabetic patients.   Diabetic gangrene is still only too prevalent and if the infection is extensive thigh amputations are the rule.   Occasionally both legs are thus lost.   More often, with advanced arteriosclerosis and doubtful prognosis, the question must be decided whether to amputate at once or spend months in a risky attempt to save the limb.   If a marked gain of assimilative power could be expected from the removal of one or both legs there are cases in which this would determine the choice.   On the other hand, if there is danger of any lowering of assimilation from the removal of large masses of muscle this a so should be known.

Theoretically it would seem possible that there is a quantitative relationship between the pancreas and the mass of body tissue which it must supply with hormone.   As previously remarked, it must be impossible for the pancreas of a mouse to supply the body of an elephant.   It is conceivable that the effects of over and undernutrition may be due to changes in the mass as well as the activity of living protoplasm, according to one well-known theory.   This involves also the question whether the pancreatic function pertains to the endogenous or the exogenous metabolism in the sense of Folin.   Determinations of the urinary creatinin and uric acid in human patients would have been simple and instructive, but were prevented by circumstances; and those actually begun in dogs failed by reason of the difficulties already mentioned (in that the dogs chosen for the purpose prove unsuitable or meet with accidents), and repetition was not possible. It is evident, however, that sudden loss of a considerable bulk of cells carries away everything pertaining to these cells in both mass and metabolism, and the effects can be compared with those of a similar reduction of weight resulting from diet.   There is some possibility that the effect may be much greater.   Another possibility is that the loss of important masses of sugar-burning tissue, especially muscles, may lower the total capacity for sugar utilization.

Opportunities have occurred for the observation of dogs (both normal ones and those rendered diabetic by removal of portions of the pancreas), that have lost large amounts of body tissue suddenly. In one instance the loss was over 20 per cent. of the body weight. Tests made on these dogs by means of intravenous injection of sugar showed no definitely increased tolerance for carbohydrates in any of them.

The opinion that the endocrine pancreatic function is concerned in exogenous rather than endogenous metabolism is in line with certain theoretical deductions. If it has been possible to perform the desired analyses in clinical cases there is reason to anticipate that obese patients whose body protein is protected by diet and who are merely reduced in fat will not show any reduced output of endogenous cretinin and uric acid. Yet diabetics of this type gain greatly in assimilative power under this program. Such a research would establish that the internal pancreatic function is not connected with the endogenous metabolism, so far as the latter is chemically measurable.

A practical point not covered by the animal observations is the assimilation per kilogram of the reduced body mass and the lower food requirement of a patient who has lost one or both legs. An indirect benefit to the diabetes from this fact can scarcely be doubted, but the point has no theoretical significance in the present connection, and as a practical measure it is scarcely to be mentioned in comparison with the therapeutic method of reducing the metabolism by undernutrition.

CONCLUSIONS.

1. The assimilative power of diabetic animals rises and falls inversely with the body weight. This change is established on different diets, which exclude the supposition that it is due merely to variations in either glycogen or protein, and which prove that it is produced also by the feeding and deposit of a non-sugar-forming material, namely fat.

2. When considerable masses of active tissue, especially muscle, are suddenly lost, as in removal of limb by amputation, the effects upon the assimilation are negligible compared with those of similar losses of weight produced by undernutrition. It may therefore be

concluded that the effects of undernutrition are not due to a reduction of active protoplasm but rather to a reduction of food supplies and metabolism.   Though there must necessarily be some relation between the mass of pancreas and the mass of body cells which it can supply with hormone, this study indicates a direct quantitative participation of this hormone in the metabolism of matter, and furthermore in exogenous rather than endogenous metabolism.   In other words, the pancreatic function is not appreciably spared when the same quantity of food is metabolized by a reduced number of cells, but rather when the same number of cells metabolize a reduced quantity of food materials.

3. This principle is important clinically in that undernutrition should be continued to the point of relieving the pancreatic function from overstrain revealed by the most delicate tests, particularly hyperglycemia.   With extremely few exceptions in human patients the curve of rising tolerance intersects the curve of falling weight at some level on which life can be maintained.   Lack of thoroughness in relieving the pancreatic function is the chief cause of continued deterioration of this function and the consequent choice between coma and starvation.   The present investigation has aimed at something more than an empiric treatment, namely, the valid proof of a quantitative relation between the internal pancreatic function and the total body weight and metabolism.   Any theories of the nature of this function or of diabetes must take account of the fact that the islands of Langerhans are concerned not merely in the combustion of sugar or storage of glycogen, but also in the maintenance of the general tissues and reserves.   In some manner an increased supply of fat or formation of adipose tissue imposes a burden on the island function, and reduction of any kind of food or of the body weight reduces the demand upon this function.   This fact seems to indicate that the island hormone has both a catabolic and an anabolic role.[9]

As the diabetic deficiency is so much more prominent with regard to carbohydrate, it is possible that the function is directly related to this alone, and that other foods are concerned only indirectly through

[9] Taylor, A. E: Tr. Coll. Phys., Philadelphia, 1916, xxxviii, 254.   Cf. Rockefeller Institute Monograph, 1919, No. 11, p. 128.

their influence upon carbohydrate metabolism, though this point remains unproved. Whether the relation be direct or indirect, the status of the pancreatic hormone in general metabolism and of the disturbance of general metabolism in diabetes is sufficiently important that treatment must be directed to it and is seldom successful if limited to restriction of carbohydrate alone.

EXPERIMENTAL STUDIES IN DIABETES.

SERIES II. THE INTERNAL PANCREATIC FUNCTION IN RELATION TO BODY MASS AND METABOLISM.

3. THE EFFECTS OF EXERCISE.

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AND

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The preceding paper dealt particularly with the metabolism of matter, in the form of changes in the food supply and bodily reserves. The present paper is devoted to the metabolism of energy as affected by exercise. It is recognized that the distinction is imperfect, since the energy of exercise must be furnished by combustion of material, which either demands increased food or causes undernutrition. Nevertheless, there is a difference, since the mere presence of stores of reserve fat was found to alter the assimilation, while exercise represents not only increased metabolism but also metabolism under a special stimulus.

Of the two factors chiefly concerned in carbohydrate combustion, one, the pancreas, was surgically reduced in Paper 1, and the other, the muscles, in Paper 2. Long-continued exercise builds up the muscles in mass and function. Athletes have a higher than average metabolism,[1] and the basal metabolism of a normal dog may fall as much as 16 per cent. in consequence of prolonged cage life.[2] As the dogs used in these experiments had mostly undergone many months of cage life, it is probable that the muscular factor in metabolism was considerably augmented by the exercise, and observations were therefore possible as to how far the development of this factor might compensate for deficiency of the pancreatic factor.

Mention has been made elsewhere[3] of the early clinical work which indicated that exercise aids sugar combustion and diminishes gly-

cosuria in mild diabetes, but is ineffective or even harmful in severe diabetes. Moraczewski[4] found that exercise increased the blood sugar of normal persons and diabetics, while at the same time improved assimilation was manifest by a diminution of glycosuria. Mackenzie[5] found a rise of 0.085 per cent. or less in the blood sugar of fasting totally depancreatized dogs with exercise, and a fall of 0.1 per cent. or less with exercise in depancreatized dogs receiving 200 gm. of meat and bread daily. The exercise periods were twenty to thirty minutes, and apparently irregularities of absorption and diuresis were not excluded as possible causes of the variations.

A preliminary outline of the present investigation and some observations on the application in clinical treatment have already been

TABLE I.

Dog C3–47.

Normal. Weight, 15.4 Kgm. The Effect of Exercise on Normal Fasting Dog.

Time	Plasma sugar, per cent.	Hb., per cent.	Urine.		Rectal temperature, °F.
			Volume, c.c.	Glucose, per cent	
1 hr. before exercise.............	0.115	112	..	0	101.4
Immediately before exercise......	0.117	110	10	0	101.6
After 15 min. exercise............	0.135	111	..	..	102.7
After ½ hr. exercise..............	0.176	111	..	..	104.1
After 1 hr. exercise..............	0.133	111	4	0	104.6
After resting 2 hrs...............	0.139	111	25	0	101.4

published.[6] In the experiments here to be described, dogs were exercised under various conditions of fasting, feeding and intravenous glucose injections. The exercise consisted in hard running on a treadmill, driven entirely by the dog, with no measurement of the amount of work done. The animals mostly enjoyed the running, and it was considered sufficient that they were kept going at full speed for the periods specified. The method of discontinuous intravenous injections, described elsewhere,[7] was adopted primarily with a view to the exercise experiments. The exposure of the jugular and other preparations were made some time in advance. Ordinarily, the glucose for each hour was given in the form of three injections, twenty minutes apart. With the dog ready on the treadmill, cath-

eterization, collection of the first blood sample and injection of the first dose of glucose were performed; next the animal ran for the specified time (generally fifteen minutes); a pause was made for the next blood sample and injection, and running was resumed.

The dogs were allowed water freely except in the experiments with intravenous injections. The volumes taken are omitted from the tables, because no differences in blood sugar were perceptible whether the animals chose to drink nothing or large quantities. Water was not given in the intravenous injection experiments and the animals were generally not thirsty.

TABLE II.

Dog B2-90.

Normal. Weight, 33 Kilos.

Date, 1915.	Hour.	Plasma sugar, per cent.	Hb., per cent.	Remarks.
Oct. 5	2.45 P.M.	0.111	65	Blood before exercise.
	3.30	0.154	66	After ¼ hr. of exercise; exercised to complete exhaustion.
Oct. 6	1.55 P.M.	0.111	64	Blood before exercise.
	2.00	0.154	79	After 5 min. of exercise.
	2.10	0.256	79	After 15 min. of exercise.
	2.25	0.216	68	After ½ hr. of exercise.
	2.55	0.108	60	After ½ hr. of rest.
	4.10	0.107	50	After 1¼ hrs. of rest.

The laboratory technic was the same as in the other papers, *i.e.*, Benedict's methods for sugar, the Fleischl-Miescher hemoglobin estimation, etc. The corpuscle volume was determined first in special capillary tubes of 12 cm. length, later in ordinary graduated centrifuge tubes. The blood was usually concentrated by exercise, but some allowances must be made for the limits of error in the methods.

DOG B2-79. (TABLES XI AND XII AND CHART III.)

Dog B2-79, as described in the preceding paper, was fattened so as to produce steady aggravation of the diabetes, while the successive recurrences of glycosuria were checked by reduction of protein and

TABLE III.

Dog C3-49.

Normal Weight 16.9 Kgm. Comparison on Normal Fasting Dog of Exercise, Exercise Plus Intravenous Glucose Injections, and Intravenous Glucose Injections Alone.

Time.	Plasma sugar, per cent.			Hemoglobin, per cent.			Urine volume, c c.			Urine glucose, per cent.			Rectal temperature. °F.*			Remarks.
	I.	II.	III.	I.	II.	III.	I.	II.	III.	I.	II.	III.	I.	II.	III.	
Before injection...........	0.133	0.106	0.119	111	99	94	..	..	..	0	0	0	102.1	102.7	102.7	Exercise started.
¼ hr. after 1st injection...	0.154	0.166	0.167	120	94	80	..	20	10	..	1.00	1.54	103.8	104.6	102.8	
¼ hr. after 2d injection...	0.172	0.142	0.154	116	96	78	..	20	2	..	1.49	++	104.7	105.6	103.4	Exercise stopped.
¼ hr. after 3d injection...	0.125	0.122	0.134	110	98	76	15	25	55	0	++	0.15	..	..	..	
½ hr. after 3d injection...	..	0.100	..	..	95	..	..	10	..	..	0	..	104.9	104.2	103.4	
¾ hr. after 3d injection...	..	0.133	..	..	95	..	..	25	..	..	0	..	..	104.7	..	
1 hr. after 3d injection...	..	0.095	..	..	..	..	..	15	..	..	0	..	102.0	103.0	102.3	

I, exercise on fasting dog; II, exercise with intravenous glucose injections 1 gm. per kgm. per hr.; III, intravenous glucose injections 1 gm. per kgm. per hr. without exercise.

* High initial temperature due to hot weather.

TABLE IV.

Dog C3-54.

Normal. Weight, 19 Kgm. Comparison of Exercise, Exercise with Intravenous Glucose Injections, and Intravenous Glucose Injections Alone.

Time.	Plasma sugar, per cent.			CO_2 volume, per cent.			Hemoglobin, per cent.			Urine volume, c c.			Urine glucose, per cent.			Rectal temperature, °F.			Remarks.
	I.	II.	III.	I	II	III	I.	II.	III.	I	II.	III	I	II.	III	I.	II.	III.	
Before injection	0.128	0.111	0.115				120	112	114				0	0	0	101.5	101.3	101.9	Exercise started.
15 min. after 1st injection	0.143	0.170	0.176			40.4	115	110	82		30	40		1.22	0.63	103.0	102.8	101.8	
15 min. after 2d injection	0.120	0.100	0.222			48.1	112	109	87		35	80		0.68	0.63	103.1	102.7	101.8	
15 min. after 3d injection	0.103	0.090	0.167			46.6	108	110	76		60	110			?	103.6	102.8	102.0	
15 min. after 4th injection		0.094	0.111			44.7		112	81		109	150		0		103.2	102.7	101.8	
½ hr. after 4th injection		0.094						112			39			0		102.1	102.5		
¾ hr. after 4th injection		0.098									65			0			102.6		
15 min. after 5th injection			0.128			46.2			81			125			0			101.8	
½ hr. after 5th injection			0.105			46.2			81			63			0			101.9	
¾ hr. after 5th injection			0.121			44.7			99			54			0	101.4	101.6	101.9	

I, exercise on fasting dog; II, exercise with intravenous glucose injections 1 gm. per kgm. per hr.; III, intravenous glucose injections 1 gm. per kgm. per hr. without exercise.

TABLE V.

Dog B2–00.

Partially Depancreatized, Non-Diabetic. Weight 14 Kgm. Comparison on Partially Depancreatized Dog of Exercise, Exercise with Intravenous Glucose Injections, and Intravenous Glucose Injections Alone.

Time.	Plasma sugar, per cent.			Hemoglobin, per cent.			Urine volume, c c.			Urine glucose, per cent.			Rectal temperature, °F.			Remarks.
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	
1 hr. before injection.........	0 105	..	..	108	..	..	28	..	..	..	..	..				
Immediately before.........	0 125	0 108	0.105	102	104	112				0	0	0	101.5	101.5	101 3	Exercise started.
¼ hr. after 1st injection.........	0 125	0.125	..	98	97	..	..	25	..	..	0 54	0 95	103.4	103 2	..	
¼ hr. after 2d injection	0.133	0.101	0 288	95	95	98	..	19	90	..	0.56	0 50	104 2	103 8	101 0	
¼ hr. after 3d injection.........	..	0 113	0 145	..	102	91	..	70	100	..	..	..	103.7	102.8	101.6	
¼ hr. after 4th injection.........	0 105	0 089	091	98	95	93	6	120	175	0	..	0	103.1	102.7	102.2	Exercise stopped.
¾ hr. after 4th injection.........	..	0 095	088	..	92	85	..	30	25	..	0	0	102 0	102.3	102.2	
1¾ hr. after 4th injection.........	..	0 130	098	..	92	80	..	25	30	..	0	0	101.7	102.8	102.0	
After resting 2 hrs.........	0.112	..	..	95	..	..	160	..	..	0	0	0				

I, exercise on fasting dog; II, exercise with intravenous glucose injections 1 gm. per kgm. per hr.; III, intravenous glucose injections 1 gm. per kgm. per hr. without exercise.

TABLE VI.

Dog B2-01.

Non-Diabetic. Weight 14 Kgm. Comparison on Partially Depancreatized Dog of Exercise, Exercise with Intravenous Glucose Injections, and Intravenous Glucose Injections Alone.

Time.	Plasma sugar, per cent.			Hemoglobin, per cent.			Urine volume, c.c.			Urine glucose, per cent.			Rectal temperature, F.			Remarks
	I	II.	III.	I.	II.	III.	I.	II.	III	I.	II.	III.	I.	II.	III.	
1 hr. before injection..........	0.120	..	..	108			15	..	..	0						
Immediately before..........	0.125	0.099	0.111	108	101	108		..	..	0	0	0	101.4	101.2	101.3	Exercise started.
½ hr. after 1st injection........		0.102	0.167	..	97	108	..	30	44	..	0.93	0.54	102.8	103.0	102.0	
½ hr. after 2d injection........		0.127	0.176	107	97	90	..	20	55	..	0.84	0.89	103.2	102.6	102.2	
½ hr. after 3d injection........		0.118	0.173	..	94	87	..	45	90	..	0.25	0.43	104.0	103.6	102.4	
½ hr. after 4th injection........	0.150	0.094	0.154	106	90	80	10	60	100	0	0.34	+	103.8	103.0	102.3	Exercise stopped.
½ hr. after 4th injection........		0.104	0.127	..	91	82	..	20	25	..	0	0	102.2	103.4	102.4	
2½ hr. after 4th injection........		0.115	0.110	..	85	89	..	10	30	0	0	0	101.9	103.7	102.6	

I, exercise on fasting dog; II, exercise with intravenous glucose injections 1 gm. per kgm. per hr.; III, intravenous glucose injections 1 gm. per kgm. per hr.

TABLE VII.

Dog B2–02.

Non-Diabetic. Weight 10.5 Kgm. Comparison on Partially Depancreatized Dog of Exercise, Exercise with Intravenous Glucose Injections, and Intravenous Glucose Injections Alone.

Time.	Plasma sugar, per cent.			Hemoglobin, per cent.			Urine volume, c.c.			Urine glucose, per cent.			Rectal temperature. °F.			Remarks.
	I.	II.	III.	I.	II.	III.	I.	II.	III.	I.	II.	III.	I.	II.	III.	
1 hr. before injection	0.114	..	..	119			3	..	..	0	0	0	..	..	..	
Immediately before	0.115	0.105	0.111	119	120	115	..	..	..		0	0	101.6	102.0	101.8	Exercise started.
¼ hr. after 1st injection	0.100	0.170	0.209	114	120	108	..	10	10	..	1.61	2.32	102.7	103.6	102.0	
¼ hr. after 2d injection	0.111	0.118	0.271	101	116	106	..	8	25	..	0.60	2.13	103.8	104.2	101.7	
¼ hr. after 3d injection	..	0.105	0.250	..	108	97	..	45	35	..	0.25	0.64	103.5	103.8	101.6	
¼ hr. after 4th injection	0.108	0.101	0.200	101	105	104	4	50	12	0	0.35	..	103.6	104.0	101.6	Exercise stopped.
¾ hr. after 4th injection	..	0.104	0.137	..	100	103	..	15	10	..	0	0	102.7	104.0	101.4	
1¾ hr. after 4th injection	..	0.128	0.125	..	96	99	..	10	10	..	0	0	101.5	103.8	101.7	

I, exercise on fasting dog; II, exercise with intravenous glucose injections 1 gm. per kgm. per hr.; III, intravenous glucose injections, 1 gm. per kgm. per hr. without exercise.

TABLE VIII.

. *Dog B2–71.*

Weight, 16.8 Kgm. Mild Diabetes.

Regular diet 1 kgm. lung, fed evenings. Improvement of assimilation of carbohydrate test-meal by exercise.

Date, 1915.	Hour.	Plasma sugar, per cent.	Hb., per cent.	Corp. vol., per cent.	Remarks.
July 19	12.00 M.	0.099	81	28.7	Blood before exercise; fed 100 gm. bread.
	12.45 P.M.	0.100	106	45.0	After ¾ hr. of hard exercise.
	2.45	0.218	99	34.3	After resting 2 hrs.
	5.00	0.118	116	....	Ran hard most of afternoon; almost collapsed at close; no glycosuria.
July 21	12.30 P.M.	0.112	90	27.2	Before feeding 100 gm. bread. At rest.
	3.15	0.286	82	....	2¾ hr. after feeding.
	5.30	0.286	..	32 5	5 hrs. after feeding; glycosuria 3.8 gm.
July 22	11.30 A.M.	0.124	82	32.2	Before exercise; exercised for 1 hr.; fed 100 gm. bread and exercise continued for ½ hr., rested for 1½ hrs.
	3.15 P.M.	0.109	89	36.5	After ¾ hr. of exercise.
	5.30	0.118	90	33.0	After 3 hrs. of exercise; no glycosuria.
July 23	12.15 P.M.	0.104	..	33.7	Blood before feeding 100 gm. bread; at rest.
	3.15	0.286	..	25.9	3 hrs. after feeding.
	5.45	0.149	..	36 0	5½ hrs. after feeding; glycosuria 4.5 gm.
July 26	12.30 P.M.	0.118	82	32.1	Before feeding 50 gm. glucose; at rest.
	2.15	0.400	86	28.3	1¾ hrs. after feeding.
	4 00	0.189	93	32.1	3½ hrs. after feeding.
	5.30	0.102	80	31.8	5 hrs. after feeding; glycosuria 3.6 gm.
July 27	12.30 P.M.	0.114	94	38.4	After 1 hr. of exercise; fed 50 gm. glucose.
	2.15	0.250	80	37.6	Exercised until 1.15; at rest until 1.50; exercised until 2.15.
	·4.00	0.182	93	32.0	Ran hard all afternoon.
	5.30	0.143	104	36.0	Completely exhausted; glycosuria 0.3 gm.

TABLE IX.

Dog C3–66.

Weight, 20 Kgm. Severe Diabetes. The Effect of Exercise on Fasting Diabetic Dog.

Time.	Plasma sugar, per cent.	Hb , per cent.	CO_2 vol. per cent.	Urine.		D:N ratio.
				Vol. c.c.	Glucose per cent.	
Immediately before exercise........	0.358	90	46.2	..	1.49	0.71
After ½ hr. exercise................	0.370	90	38.5			
After 1 hr. exercise................	0.455	84	34.7	2	Slight	..
After resting 1 hr..................	0.435	73	46.2	34	3.13	4.05

TABLE X.

Dog B2-89.

Weight, 13.2 Kgm. Severe Diabetes. Comparison on Partially Depancreatized Dog, Severely Diabetic, of Exercise with Intravenous Glucose Injections and Intravenous Glucose Injections Alone.

Time.	Plasma sugar, per cent		Hemoglobin, per cent.		Urine volume, c.c.		Urine sugar, per cent.		Rectal temperature, °F.		Remarks.
	I.	II	I	II.	I	II.	I.	II	I	II.	
Before injection.............	0.133	0·122	117	112	..	..	0	0	101 8	101.2	Exercise started.
¼ hr. after 1st injection......	0 264	0.256	109	111	25	16	2.22	4 00	104 0	103.8	
¼ hr. after 2d injection......	0.400	0 333	93	100	52	30	3 30	5 00	104 0	104.9	
½ hr. after 2d injection......	0 264	0.278	100	74	10	9	3 30	3 83	105 5	104.5	
¾ hr. after 2d injection......	0 218	0 250	105	76	5	4	++	0 40	105 0	103 9	
1 hr. after 2d injection......	0.103	0 141	98	83	5	2	+	++	104 4	103 4	Exercise stopped.
1½ hr. after 2d injection.....	0 131	0.122	86	78	6	4	0	+++	102 5	104.2	

I, exercise with intravenous glucose injections, 1 gm. per kgm. per hr.; II, intravenous glucose injections, 1 gm. per kgm. per hr. without exercise.

· TABLE XI.

Dog B2-79.

Weight 15 Kgm.   Increasingly Severe Diabetes.

Date.	Hour.	Plasma sugar, per cent.	Hb., per cent	Corp. vol., per cent.	CO₂ vol., per cent.	Urine sugar, per cent.	Remarks.
1915. July 17	10.30 A.M.	0 133	100	46.2	..	..	Blood taken before exercise.
	12.30 P.M.	0.100	120	49 0	..	..	After exercising hard for 2 hrs.
July 23	2.30 P.M.	0.109	105	45.3	..	..	Before exercise.
	6.00	0 105	125	49.5	..	..	After 3½ hrs. hard exercise.
Sept. 25	12.15 P.M.	0 169	110	50 0	..	..	After 5 min. of exercise.
	1 10	0.143	110	52 0	..	..	After 1 hr. of exercise.
Dec. 1	10.40 A.M.	0 278	111	43.5	..	1.03	Before exercise; fasting.
	1.40 P.M.	0.278	104	40 0	..	0 25	Immediately before exercise.
	1.50	0.313	108	48 5	..	..	After 10 min. of exercise.
	2.05	0.159	98	..	..	..	After 25 min. of exercise.
	2.40	0.143	97	..	..	..	After 1 hr. of exercise.
	3.40	0.256	97	41 0	..	Faint	After 2 hrs. of exercise.
Dec. 6	1.50 P.M.	0.200	..	..	..	0	Before exercise fed 500 gm. lung; allowed to rest ½ hr. before exercise.
	4.50 P.M.	0.208	..	..	..	Slight	After 2½ hrs. of exercise.
1916. Feb. 3	1.50 P.M.	0.278	95	..	..	Very faint	1 hr. before exercise; fasting.
	2.50	0.334	95	..	..	Very faint	Immediately before exercise.
	3.05	0.264	98	..	..	..	After 15 min. of exercise.
	3.20	0.304	103	..	..	..	After ½ hr. of exercise.
	3.50	0.278	105	..	..	Slight	After 1 hr. of exercise.
	4.50	0 294	..	..	..	0	After resting 1 hr.
Mar. 30	3 20 P.M.	0.455	90	..	42.3	0 61	Before exercise; fasting; D:N ratio 1.90.
	3.50	0.312	82	..	31.9	..	After ½ hr. of exercise.
	4.20	0.435	72	..	34.7	0 97	After 1 hr. of exercise; D:N ratio 6.45.
	5.20	0.435	72	..	42.3	0.78	After resting 1 hr.; D:N ratio 2.30.

TABLE XII.

Dog B2-79.

Time.	Nov. 8.			Nov. 11.			Nov. 12.			Nov. 26.	Nov. 29.
	Plasma sugar, per cent.	Hb., per cent.	Corp. vol., per cent.	Plasma sugar, per cent.	Hb., per cent.	Corp. vol., per cent.	Plasma sugar, per cent.	Hb., per cent.	Corp. vol. per cent.	Plasma sugar, per cent.	Plasma sugar, per cent.
Before exercise or food	0.125	116	53.0	0.161	115	57.8	0.115	109	40.0	0.156	0.154
¾ hr. after food	0.142	110	50.0	0.133	115	50.0	0.115	105	41.0	..	..
1 hr. after food	0.184	112	51.0	0.142	113	44.0	..	..	..	..	..
1¾ hr. after food	0.167	114	..	0.130	109	51.0	0.108	105	44.5	..	..
3 hr. after food	0.128	112	58.0	0.139	100	47.5	0.111	..	..	0.182	..
4 hr. after food	0.147	114	51.0	..	..	..	..	..	..	..	..
5 hr. after food	0.141	109	65.0	..	..	..	..	..	..	0.250	0.208

A, fed 500 gm. lung; Nov. 8, at rest; Nov. 11 and 12, with exercise. B, fed 1 kgm. lung; Nov. 26, at rest; Nov. 29, with exercise.

substitution of fat. Table XI illustrates the effects of exercise in the different stages of progress on days without feeding. On July 17 and 23 the plasma sugar was low and was reduced a trifle by exercise. On September 25 hyperglycemia was present but was lowered by exercise. On December 1 the hyperglycemia was greater; it rose sharply with ten minutes of exercise, then fell during the first hour, but showed a rising tendency at the end of the second hour. On December 6 exercise seemed to prevent the usual rise of blood sugar after protein feeding, but it is possible that digestion was delayed. On February 3 fluctuations of the hyperglycemia occurred, but any effect of exercise is doubtful. On March 30 there was a fall of plasma sugar, particularly after thirty minutes of exercise, but the actual effect was injury rather than benefit, as shown in the extra sweeping out of sugar represented in the D:N ratios.

Table XII shows protein-feeding experiments on the same dog during November, 1915. A real improvement of assimilation with exercise at this stage, as opposed to a mere slowing of food absorption, is indicated by the fact that the plasma sugar elevations were longer as well as higher on rest days, and also on a second exercise day (November 12) were lower than on the day before. There was no glycosuria.

DOG B2-80. (TABLE XIII AND CHART V.)

Dog B2-80, as described in the preceding paper, progressed to severe diabetes and acidosis on excessive feeding, particularly with fat. Toward the end exercise was tried on several days. The dog was strong and willing and ran hard. The plasma sugar was increased rather than diminished. The rectal temperature rose as usual above 104°F. during exercise. Acidosis was present throughout, as indicated by nitroprusside reactions in blood and urine and rather low plasma bicarbonate values, but the former did not increase and for some reason the bicarbonate concentration actually rose with the running. There were also no coma symptoms even when the dog ran to exhaustion. This was the more remarkable because coma was so near. No exercise was used after August 8 and the condition appeared unchanged. Vomiting began on August 11, and coma on August 13.

TABLE XIII.

Dog B2–80.

Weight, 17 Kgm.

Date.	Hour.	Plasma sugar, per cent.	Hb. per cent.	Corp. vol., per cent.	Urine. Vol., c.c.	Urine. Sugar, per cent.	Remarks.
July 23	2.30 P.M.	0.263	114	49.0	..	..	Before exercise; fasting.
	6.15	0.270	120	55 5	..	..	After 3¾ hrs. of hard exercise.
July 26	11.30 A.M.	0.208	118	50 3	..	..	Before exercise.
	12.30 P.M.	0.208	125	53.6	..	..	After 1 hr. of exercise.
Aug. 8	10.50 A.M.	0.294	103	55.0	..	0 66	Before exercise.
	11 00	0.294	101	56.0	6	0.56	After 10 min. of hard exercise.
	12.20 P.M.	0.322	94	61.0	9	1.67	After resting 1 hr. 20 min.
	12.50	0 333	105	64.5	5	1.23	After ½ hr. of very hard exercise; exhausted.
	4.30	0.303	104	53.4	16	1.25	After resting 3 hrs. 40 min.

TABLE XIV.

Dog C3–18.

Weight, 15 Kgm.

Date.	Hour.	Plasma sugar, per cent.	Urine sugar, gm in 24 hours.	Remarks.
1915. Nov. 15	10.35 A.M.	0.188	2.94	Blood taken before exercise; fed usual diet.
	11.30	0.256	....	After 20 min. of exercise; rather weak; cannot be exercised long at a time.
	2.50 P.M.	0.555	....	After resting 3½ hrs.
Nov. 16	9.45 A.M.	0.238	0	Before exercise; fasting.
	10.15	0.222	....	After ½ hr. of exercise; exercise stopped.
	10.35	0.222	....	After resting ½ hr.; exercise started.
	11.05	0.238	....	After ½ hr. of exercise; urine for the period 12 c.c.; negative sugar.
Nov. 26	2.00 P.M.	0.186	0	Before exercise; fed usual diet.
	4.30	0.182	....	After 2½ hrs. of exercise.
Nov. 27	1.00 P.M.	0.208	0	Before feeding; fed usual diet; at rest.
	3.30 P.M.	0.262	....	After resting 2½ hrs.
Nov. 29	1.55 P.M.	0.218	0	Before exercise.
	3.30	0.147	....	After 1½ hrs. of exercise.
Dec. 1	10.10 A.M.	0.196	0	Fasting for 36 hrs.; blood taken before exercise.
	12.10 P.M.	0.162	....	After two hrs. of exercise.
	1.45	0.196	....	After resting 1½ hrs.; urine for the period 95 c.c.; negative sugar.

DOG C3–18. (TABLE XIV AND CHART V.)

Dog C3–18 was a graft animal with diabetes, like dog C3–02. The period covered in Table XIV represents gain in tolerance, apparently due in part to exercise. There was a fixed diet of beef-lung and pancreas, on which the body weight declined by about three-fourths of

TABLE XV

Dog C3–19.

Severe Diabetes.

Date.	Hour.	Plasma sugar, per cent.	Hb., per cent.	Corp. vol., per cent.	Urine sugar, per cent.	Remarks.
Aug. 11	3.00 P.M.	0.250	87	36.7	0	Before feeding 500 gm. lung; at rest.
	6.00	0.276	80	35.2	3.84	3 hrs. after feeding.
	11.30	0.303	90	34.6	6.66	8½ hrs. after feeding; glucose excreted 6.4 gm.
Aug. 12	12 00 M.	0.294	85	35.0	2.22	Before exercise; fed 500 gm. lung.
	12.40 P.M.	0.345	..	..	..	Exercised from 12.15 to 12.40; glucose excreted before exercise 3.5 gm.
	1.00	0.286	98	40.0	2.27	Exercised from 12.45 to 1; glucose excreted after exercise 2.3 gm.
	3.00	0.263	88	31.5	1.58	After alternate rest and exercise periods.
	8.45	0.192	84	30.2	0.75	Exercised until 6.30, rest thereafter.

Dog C3–19, weighing 11.1 kilos, tolerated 500 gm. lung on August 9 without glycosuria, but showed glycosuria of 1.1 gm. on August 10. On the two following days rest and exercise were compared with results shown in Table XV. The increasing glycosuria was arrested by exercise, inasmuch as 3.5 gm. glucose was excreted during the forenoon of August 12 without exercise or food, and only 2.3 gm. during the remainder of the twenty-four hours with feeding and exercise.

a kilo during this period, so that part of the improvement of assimilation may be attributed to undernutrition; but the exercise must also have contributed toward the undernutrition.

On November 15 exercise failed to prevent a great rise of blood sugar after feeding or stop the existing glycosuria. November 16 the initial blood sugar was higher but did not rise during one and a half hours. November 17 the urine was free from sugar. With

continuous rest thereafter, except for a single exercise day on November 19, glycosuria remained absent through November 23. It was present on November 24, 25 and 26 and ceased with a single exercise day on November 26. The table shows a rise of blood sugar after feeding on the rest day November 27 and none on the exercise day November 26. Glycosuria remained absent. On November 29 exercise reduced the blood sugar. The dog was fed that evening, but not on November 30. On December 1 the blood sugar fell during two hours of exercise and rose during the ensuing one and a half hours of rest.

The record suggests not only that exercise facilitated assimilation, but also that it was more effective after the tolerance had been· improved by undernutrition. (See also Chart V.)

DOG c3–22. (TABLE XVI AND CHART V.)

Dog C3–22. Severe diabetes, controlled four months by diet, first 1 kilo of lung, then 400 gm. lung and 200 gm. suet, then 300 gm. lung and 200 gm. suet, then 200 gm. lung and 200 gm. suet. The recurrences of glycosuria were checked each time by the reduction of protein, but the usual gradual aggravation occurred on the high calory diets, and at the time of these experiments glycosuria was returning on the diet last mentioned. Exercise was begun November 12 in the attempt to stop the downward progress. The weight was then 11.5 kgm.

November 12, hyperglycemia increased after feeding notwithstanding exercise, but by the next day there was reduction of hyperglycemia and glycosuria. Both were found higher on November 15, after the preceding rest day, but an alternation of rest and exercise (November 15) showed that exercise reduced the blood sugar. On November 16 the fasting blood sugar was lower but was not appreciably reduced by exercise. Hard exercise was then given every day, both before and after feeding, through November 19; no blood sugar analyses were made, but glycosuria remained absent.

Beginning November 20 the dog remained at rest. Traces of glycosuria began November 25 and increased the next day. On that day (November 26) exercise was given; the blood sugar was lowered and the glycosuria remained too small to titrate. November

27 was a control day without exercise and showed a marked rise of blood and urine sugars as compared with November 26.

TABLE XVI.

Dog C3–22.

Normal Weight, 13.4 Kgm.   Severe Diabetes.

Date.	Hour.	Plasma sugar, per cent.	Urine sugar, gm in 24 hours.	Remarks.
1915.				
Nov. 12	10 15 A.M.	0 222	Faint	Before exercise; fed usual diet.
	11 20	0.238	....	After 1 hr. of exercise.
	3 00 P.M.	0 322	3.1	After 3½ hrs. of rest.
Nov. 13	2.00 P.M.	0 145	1.6	Before exercise; fasting.
	3.00	0 125	....	After 1 hr. of exercise.
	4 00	0 125	....	After 2 hrs. of exercise.
Nov. 14	....	....	Slight	Rest day.
Nov. 15	10.25 A.M.	0 200	1.7	Before exercise; fed usual diet.
	12.00 M.	0 250	....	After 1 hr. of exercise.
	1.30 P.M.	0 263	....	After resting 1½ hrs.
	2 30	0 182	....	After 1 hr. of exercise.
	3 00	0.170	....	After 1½ hrs. of exercise.
	4.20	0 257	....	After resting 1 hr. 20 min.
Nov. 16	9.35 A.M.	0 159	Faint	Before exercise; fasting.
	10 35	0 154	....	After 1 hr. of exercise.
	11 35	0 154	....	After 2 hrs. of exercise.
Nov. 26	2 05 P.M.	0 227	Faint	Before exercise; fed usual diet.
	4.30	0 186	....	After 2¼ hrs. of exercise.
Nov. 27	10.50 A.M.	0 256	1 3	Fed usual diet; no exercise.
	2.50 P.M.	0 400	....	4 hrs. after feeding.
Nov. 28	....	....	1.1	Exercise day.
Nov. 29	1.55 P.M.	0 227	0 3	Before exercise; fasting.
	3.30	0 170	....	After 1½ hrs. of exercise.
Nov. 30	....	....	....	Fast day.
Dec. 1	10.10 A.M.	0 200	0	Before exercise; fasting.
	12.10 P.M.	0.128	....	After 2 hrs. of exercise.
	1.45	0 200	....	After resting 1 hr. 35 min.
Dec. 6	1.50 P.M.	0.313	1 2	Before exercise; fed usual diet; rested ½ hr. before beginning exercise.
	4.50	0.500	....	After 2½ hrs. of exercise.
Dec. 7	....	....	2.7	Rest day.
Dec. 8	....	....	3.5	Rest day.
Dec. 9	....	....	13.6	Rest day.

Exercise was then continued and reduced the glycosuria but failed to abolish it until the fast day of November 30. The regular diet of 200 gm. lung and 200 gm. suet then continued, with the hardest possible exercise occupying the greater part of every day. Nevertheless, traces of glycosuria began December 2 and gradually increased to 1.2 gm. on December 6. Exercise thus finally failed to suppress hyperglycemia and glycosuria, but evidently restrained them somewhat, as shown by the rapid increase with discontinuance of exercise following December 6.

TABLE XVII.

Dog C3–02.

Subcutaneous Graft.

Date.	Hour.	Plasma sugar, per cent.	Hb., per cent.	Rectal temp., °F.	Remarks.
1915. Aug. 10	11.30 A.M.	0.120	95		Blood taken; dog allowed to rest quietly in cage.
	3.30 P.M.	0.145	91	..	Blood taken after resting 4 hrs.; fed 600 gm. lung, 600 gm. raw pancreas and 100 gm. suet.
	6.00	0.286	100	..	Blood taken 2½ hrs. after eating.
Aug. 11	12.25 P.M.	0.139	88	102.4	Blood taken before exercise.
	1.00	0.154	97	103.5	After ½ hr. of exercise.
	3.30	0.164	98	..	After 2½ hrs. of rest; fed same diet as on Aug. 10.
	4.30	0.151	..	104.8	After 1 hr. of exercise.
	6.00	0.164	..	105.5	After 2½ hrs. of exercise.

DOG C3–02. (TABLE XVII.)

Dog C3–02 was an animal with moderate diabetes, having no pancreas tissue except a subcutaneous graft, of which the pedicle had been cut so as to isolate it from all intra-abdominal connections. Table XVII shows that exercise had the usual effect in restraining the rise of blood sugar after feeding, and thus excludes any nervous influence upon the pancreas as the cause of this action.

TABLE XVIII.

Dog C3-60.

The Effect of Exercise on Totally Depancreatized Dogs.

Time.	Blood.						Urine.					
	Plasma sugar, per cent.		CO_2, volume per cent.		Nitroprusside reaction.		Volume, c.c.		Sugar, per cent.		D:N ratio.	
	Feb. 24.	Feb. 25.	Feb. 24	Feb. 25	Feb. 24	Feb. 25.	Feb. 24	Feb. 25	Feb. 24	Feb. 25	Feb. 24	Feb. 25.
Before exercise	0.264	0.270	40.0	46.2	..	Faint	..	..	4.76	3.70	2.22	1.54
After ½ hr. exercise	0.294	0.333	39.0	38.5	..	Faint	0	..	..	..	..	..
After ½ hr. exercise	0.346	0.358	38.1	33.8	..	Faint	5	13	3.40	0.95	..	3.28

Dogs C3-65 and C3-76.

Time.	Blood.								Urine.					
	Plasma sugar, per cent.		Hemoglobin, per cent.		CO_2, volume per cent.		Total acetone, mgm per 100 c.c.		Volume, c.c.		Sugar, per cent.		D:N ratio.	
	Dog C3-65.	Dog C3-76.	Dog C3-65.	Dog C3-76.	Dog C3-65	Dog C3-76.	Dog C3-65.	Dog C3-76.	Dog C3-65	Dog C3-76	Dog C3-65.	Dog C3-76.	Dog C3-65.	Dog C3-76.
Before exercise	0.385	0.500	94	95	51.0	44.3	Mod.	33.1	·	..	3.12	2.21	1.75	2.50
After ½ hr. exercise	0.455	..	89	..	44.3	..	Slight	..	4	..	2.38	2.67	1.88	3.45
After 1 hr. exercise	..	0.500	..	84	..	38.5	..	48.3	0	5	..	4.00	..	..
After 1 hr. rest	..	0.500	..	89	..	44.3	..	26.6	..	32	..	..	..	..

TABLE XIX.

Dog C3-53.

Weight 12.3 Kgm. The Effect of Exercise on a Phlorizinized Dog.

Time.	Dec. 27, before giving phlorizin.				Dec. 28, phlorizin and exercise.				Dec. 29, phlorizin and exercise.				Dec. 30, phlorizin without exercise.			
	Plasma sugar, per cent.	Hb, per cent.	Urine.		Plasma sugar, per cent.	Hb., per cent.	Urine.		Plasma sugar, per cent	Hb., per cent.	Urine.		Plasma sugar, per cent.	Hb, per cent.	Urine.	
			Vol., c.c.	Sugar, per cent.			Vol., c.c.	Sugar, per cent.			Vol., c.c.	Sugar, per cent.			Vol., c.c.	Sugar per cent.
1 hr. before exercise	0.111	112	45	0	0.097	106	28	7.15	0.077	101	290	7.82	0.095	86	124	6.60
Immediately before exercise					0.112	106	25	7.700	0.092	110	25	8.25	0.083	86	14	6.72
After 15 min. of exercise					0.159	110	..	..	0.082	104						
After ½ hr. of exercise					0.119	108	..	..	0.119	100						
After 1 hr. of exercise					0.125	95	20	9.10	0.132	108	25	10.0				
After resting 2 hrs.					0.118	92	55	7.70	0.100	95	175	2.5	0.081*			
After resting 5 hrs.					.108	106	50	7.70	0.072	..	75	5.5			67*	6.62*

* Taken 2½ hours after previous blood, dog at rest all the time.

TABLE XX.

Comparison on a Phlorizinized Dog of Intravenous Sugar Injections, Intravenous Sugar Injections with Exercise, and Exercise Alone.

	Intravenous sugar injections.					Intravenous sugar injections with exercise.				Exercise without injections.					Remarks.
	Plasma sugar, per cent.	Hb., per cent.	Urine Vol., c.c.	Urine Sugar, per cent	D:N.	Plasma sugar, per cent.	Hb., per cent.	Urine Vol., c.c.	Urine Sugar, per cent.	Plasma sugar, per cent.	Hb., per cent.	Urine Vol., c.c.	Urine Sugar, per cent.	D:N.	
Before injection or exercise......	0 095	95	65	1.32	4.02	0 056	112	33	5.40	0 080	95	54	3.70	3 80	Exercise started.
15 min. after 1st injection........	0 178	80	110	3 03	..	0 181	..	14	6.25	0 095	108	..	..	..	After ½ hr. exercise.
15 min. after 2d injection........	0 193	80	106	3 12	..	0 161	90	24	4 00	..	..	..	..	..	
15 min. after 3d injection........	0 214	80	130	1.88	..	0 250	98	19	2.50	0 087	105	..	3.57	4 26	After 1 hr. exercise.
½ hr. after 3d injection.........	0 193	77	81	1.53	..	0 118	92	8	2 78	..	..	..	..	..	
45 min. after 3d injection........	0 143	77	62	1 51	..	..	..	..	..	..	..	..	..	..	

TABLE XXI.

Dog C3-57.

Weight 14 Kgm. Phlorizinized.

Time.	Blood.				Urine.				Remarks.
	Plasma sugar, per cent.	Hb., per cent.	CO₂, vol. per cent.	Nitro-prusside reaction.	Volume c.c.	Sugar per cent.	D:N. ratio.	Nitro-prusside reaction	
Mar. 2.									
8.00 A.M.	0.065	92	41.4	Faint	..	3.03	3.24	Very heavy	
11.00 A.M.	0.080	95	41.4	Faint	54	3.70	3.80	Very heavy	Started exercise.
11.30 A.M.	0.095	108	33.8	Faint					Exercise finished; some urine lost on treadmill.
12.00 M.	0.087	105	36.6	Slight	6	3.57	4.26	Heavy	
Mar. 3.									
10.40 A.M.	0.056	112	40.4	Slight	..	5.90	..	Mod.	10.50 finished 1st intravenous glucose 0.5 gm. per kgm. and exercise started.
11.05 A.M.	0.182	..	39.5	0	14	6.25	..	Faint	11.20 finished 2d injection.
11.40 A.M.	0.161	90	33.8	0	24	4.00	..	0	11.58 finished 3d injection.
12.15 P.M.	0.250	98	36.6	0	19	2.50	..	0	
12.30 P.M.	0.118	92	38.5	0	8	2.78	..	0	Urine lost on treadmill; exercise stopped.

Note on Tables XIX, XX and XXI.

These fasting dogs received 1 gm. phlorizin in oil suspension subcutaneously daily. Mobilization of extra sugar is indicated by the rise of blood sugar and also of the D:N ratios, according to the principles made familiar by Lusk. It may further be noted (Dog C3-57) that the absolute sugar excretion was diminished, at least for the brief experimental period, by reduction of the volume of urine by exercise. It was not determined whether the intravenously injected glucose was quantitatively excreted, but the nitroprusside reactions in blood plasma and urine were cleared up.

THERAPEUTIC RESULTS OF LONG-CONTINUED EXERCISE.

DOG B2–86.

Dog B2–86 was described in Paper 2 of Series I[8] as an animal in which prolonged maximal feeding of starch and sugar barely failed to break down the tolerance. In this and a series of similar dogs the attempt was made to keep up the failing appetite with exercise, in the hope that the tolerance might finally break down, but the existing glycosuria merely cleared up more quickly and remained absent. Prolonged overfeeding with exercise therefore failed to produce diabetes in dogs which were non-diabetic on feeding alone.

DOG B2–43.   (TABLE XXII.)

Dog B2–43 was an animal with mild diabetes, free from glycosuria on a diet of 1 kilo of lung, fed evenings, but with a limited tolerance for bread, as shown in the experiments in Paper 2 of Series I. In July, 1915, experiments were performed, indicating a better assimilation of carbohydrate test-meals on exercise days than on rest days, as shown in Table XXII. The weight during this time was stationary at 10.5 kilos.

After a few experiments with cold, as described in a later paper, the dog was left at rest, and a beneficial after-effect of the exercise was indicated by the continued absence of glycosuria on the diet of 1 kilo lung and 100 gm. bread. On this diet without exercise the weight rose to 12 kilos by August 31, when glycosuria began with 0.7 per cent. sugar in 450 c.c. urine, increasing to 1.4 per cent. in 419 c.c. urine on September 1. With exercise this glycosuria immediately dropped to faint traces and ceased September 2. Exercise was then continued to the extent of an hour or two daily after the carbohydrate feeding; glycosuria thus remained absent while the weight rose to 12.8 kilos by September 10.

With omission of exercise, September 10 to 17, there was daily glycosuria from 0.16 to 0.9 per cent., while the weight rose to 14 kilos.

Heavy exercise was then resumed and at the same time the bread increased to 150 gm. daily. The weight thus gradually fell to 11.8 kilos on October 6 and glycosuria was continuously absent. On

TABLE XXII.

Dog B2–43.

Mild Diabetes.

Date.	Hour.	Plasma sugar, per cent.	Hb, per cent.	Corp. vol, per cent	Remarks.
July 17	9.30 A.M.	0.111	98	43.7	Started exercise; fasting.
	11.00	0.106	99	39.3	After 1½ hrs. of exercise.
July 19	12.00 M.	0.118	104	43.0	Blood taken before feeding 100 gm. bread; 12.15 started exercise.
	12.45 P.M.	0 118	83	43.0	After ½ hr. of exercise.
	2 30	0 228	95	40 0	After resting 1¾ hrs.
	5.00	0.085	98	40.5	After 2½ hrs. of exercise.
July 21	12.30 P.M.	0.133	90	43.5	Before feeding 100 gm. bread; at rest.
	3.00	0.178	96	45.0	2½ hrs. after feeding.
	5.30	0 222	..	37 1	5 hrs. after feeding.
July 22	12.30 P.M.	0 125	97	38.0	After 1 hr. of exercise, fed 100 gm. bread; exercised until 1 P.M.
	3.15	0.115	94	38.5	Rested in cage until 2.30, then started exercise; blood taken after ¾ hr. of exercise.
	5.30	0.111	96	37.5	After 3 hrs. of exercise.
July 23	12.15 P.M.	0.125	96	37.3	Before feeding 100 gm. bread; at rest.
	3.15	0.217	93	37.0	3 hrs. after feeding.
	5.45	0.133	..	35.3	5¼ hrs. after feeding.
July 24	12.00 M.	0.128	86	40 8	After 2 hrs. of exercise; fed 100 gm. of bread and by mistake 1 kgm. lung; rested remainder of day.
	3.15 P.M.	0.244	91	40.8	3¼ hrs. after feeding.
	5.30	0.270	81	38.0	5½ hrs. after feeding.
July 26	12.00 M.	0.115	83	35 0	Before feeding 50 gm. glucose.
	1.45 P.M.	0.294	92	36.5	1¾ hrs. after feeding.
	4.00	0.112	80	37.0	4 hrs. after feeding.
	5.30	0.137	88	36.0	5½ hrs. after feeding.
July 27	12.30 P.M.	0.156	95	37.0	After ¾ hrs. of exercise; 12.30 P.M. fed 50 gm. glucose.
	2.15	0.123	84	35.0	Exercise continued to 1.15, rested in cage to 2.15.
	4.30	0.107	70	45.0	
	6.00	0.100	73	37.8	Exercised from 2.15 to 6.00 P.M.

October 6 the bread was increased to 250 gm., still without glycosuria, but the dog now left most of the lung uneaten, and the weight thus fell further to 10.6 kilos on October 20.

Exercise was then stopped and 100 gm. lard added to the diet. Glycosuria remained absent, even though the weight gradually rose to 14.5 kilos by the following March. With an unlimited diet of bread only, the glycosuria then returned.

The final marked improvement was doubtless due largely to recuperation in the pancreas remnant, but this was evidently favored by exercise. Though the earlier experiments indicated that exercise favored assimilation even when the weight rose, the benefit was doubtless greater when the weight was reduced. It may be inferred that reduction of weight is beneficial even when it is produced by the increased metabolism of exercise instead of by reduction of the food supply.

DOG B2–88. (TABLES XXIII AND XXIV AND CHART IV.)

Dog B2–88 was mildly diabetic three months after operation, sugar-free on 1 kilo of beef-lung daily, but with glycosuria between 1 and 2 per cent. with 100 gm. bread. At this stage (July 12, 1915), at a weight of 12 kilos, 25 gm. bread was added to the diet and exercise begun. With increasing exercise the bread was increased to 50 gm. on July 18 and to 100 gm. on July 27 without glycosuria.

July 28 exercise was omitted and there was immediate glycosuria of 6.2 gm. in the ensuing twenty-four hours. July 29 the same diet was taken with exercise without glycosuria. There was the usual curve of hyperglycemia on July 28, while on July 29 the plasma sugar did not go above 0.128 per cent. Exercise thus permitted assimilation of 100 gm. bread, though the weight meanwhile had risen to 12.8 kilos.

As a further control to exclude a spontaneous rise of tolerance both bread and exercise were omitted for a month. Then at an increased weight of 13.6 kilos the addition of 100 gm. bread on August 30 caused glycosuria of 0.4 per cent. in 761 c.c. urine, increasing the next day to 1.2 per cent. in 724 c.c. With exercise the sugar excretion was halved on September 1 and absent September 2.

The dog was strong and fond of running and by harder exercise it was possible to increase the bread to 150 gm., though the weight rose gradually, and traces of glycosuria were present whenever the exercise was relaxed a little. By September 28 the weight had reached 14.8

kilos and the bread was increased to 200 gm. Traces of glycosuria were present during the ensuing week and were stopped by increasing exercise, so that the dog spent the greater part of every day on the treadmill. Meanwhile much of the lung of the diet was left uneaten and the weight by October 20 had fallen to 13.3 kilos.

Exercise was then stopped and a diet begun of 200 gm. bread, 100 gm. lard and only 200 gm. lung. November 5, at a weight of 14.1

<center>TABLE XXIII.</center>

<center>*Dog B2–88.*</center>

<center>*Moderate Diabetes.*</center>

Time.	Plasma sugar, per cent.					
	Nov. 9.	Nov. 10.	Nov. 11.	Nov. 12.	Nov. 18.	Nov. 27.
Before exercise or feeding...........	0.189	0.114	0.122	0.122	0.143	0.137
After ¼ hr. of exercise..............	....	....	....	0.135		
After ½ hr. of exercise..............	....	....	....	0.125		
After 1 hr. of exercise..............	....	....	....	0.112		
¼ hr. after eating...................	0.169	....	....	....	0.143	0.154
½ hr. after eating..........•.......	0.250	....	....	....	0.147	0.173
¾ hr. after eating...................	....	....	0.149		•	
1 hr. after eating...................	0.257	0.133	0.167	0.145	0.166	0.257
1¾ hr. after eating.................	....	....	0.143			
2 hrs. after eating.................	....	0.154	....	0.143		
3 hrs. after eating.................	0.257	0.200	0.137	0.156	0.222	0.333
4½ hrs. after eating................	....	....	....	0.154		

A test diet of 200 gm. bread and 100 gm. lung was fed on the six days mentioned, without glycosuria. November 9 and 27 were rest days. On November 10, 11 and 18 exercise was begun immediately after feeding, and resulted in lower blood sugar curves than on the control days. On November 12 exercise was given for one hour before feeding and the dog then left at rest after feeding. The blood sugar curve seemed to indicate a beneficial after-effect of the exercise upon assimilation.

kilos, glycosuria appeared (0.34 per cent. in 780 c.c. urine), and increased on the following day to 0.95 per. cent. in 620 c.c. It was abolished by a fast day on November 7. This control proved that the preceding absence of glycosuria had not been due to the reduced protein intake.

Exercise was then omitted except for a series of tests, most of which are shown in Tables XXIII and XXIV and Chart IV. It was evi-

TABLE XXIV.

Dog B2-88.

Moderately Diabetic. Comparison of Exercise, Exercise with Intravenous Glucose Injections, 1 Gm. per Kgm. per Hr., and Intravenous Glucose Injections Alone.

Time.	Plasma sugar, per cent.			Hemoglobin, per cent			Urine volume, c.c.			Urine glucose, per cent			Rectal temperature °F.		
	I.	II.	III.	I.	II.	III.	I.	II.	III.	I.	II.	III.	I.	II.	III.
Before injection............	0 143	0 161	0.118	..	..	107	..	..	..	0	0	0	..	..	101.6
½ hr. after 1st injection	0 156	0 200	0 200	..	..	93	..	15	120	..	0	0	..	..	101.1
½ hr. after 2d injection.......	0 189	0 200	0 200	..	..	91	..	10	64	..	0	0	..	..	101.0
½ hr. after 3d injection	..	0 164	0 232	..	..	94	..	4	73	..	0	0	..	..	101.2
½ hr. after 4th injection......	0 161	0.147	0 192	..	..	85	10	26	100	0	0	0	..	..	101.6
½ hr. after 5th injection......	..	0 126	0 200	..	..	82	..	5	106	..	0	0	..	..	101.6
½ hr. after 6th injection......	..	0 118	0 200	..	..	89	..	17	64	..	0	0	..	..	101.4
½ hr. after 7th injection......	..	0 112	0 200	..	..	91	..	20	90	..	0	0	..	..	101.5
½ hr. after 8th injection......	..	0 121	0.128	..	..	..	..	20	110	..	0	0	..	..	
½ hr. after 9th injection......	..	0 105	0.112	..	..	..	..	20	117	..	0	0	..	..	
½ hr. after 10th injection.....	..	0 133	0 121	..	..	..	..	16	..	..	0		..	..	

I, Nov. 29, 1915, exercise fasting; II, Dec. 2, exercise with intravenous glucose injections, 1 gm. per kgm. per hr.; III, Dec. 7, intravenous glucose injections, 1 gm. per kgm. per hr.

dent from these that exercise retained its power to diminish hyperglycemia even after long usage.

Subsequently, without exercise, on a diet of 200 gm. bread and 100 gm. lung with such lard as might be eaten, there was gradual increase of weight without glycosuria but with increasing hyperglycemia. April 10, 1916, at a weight of 16 kgm., glycosuria appeared and rapidly increased in a more threatening manner than before, reaching 4.5 per cent. The heaviest exercise was then unable to stop it, though it ceased promptly with omission of bread or with single fast days. April 22 to 30 glycosuria was kept absent by carbohydrate-free diet in order to give a fresh start, but with addition of the 200 gm. bread there was again heavy glycosuria in spite of exercise.

Beginning May 5 exercise and reduction of the bread ration to 100 gm. kept glycosuria absent and reduced the weight to 14.3 kgm. Nevertheless, glycosuria appeared on May 14 and 15, was temporarily checked by increased exercise, but reappeared May 18 and resisted exercise.

Exercise and carbohydrate were then omitted, and on regulated diet the weight was gradually reduced by August 16 to 13.2 kilos. Though the tolerance at this weight had formerly been so high, marked glycosuria now resulted, even after the long interval allowed for recuperation, from the addition of as. little as 25 gm. bread. With exercise this glycosuria was promptly abolished and remained absent on a diet of 200 gm. lung, 100 gm. suet and 25 gm. bread until September 12, when it reappeared at a weight of 13.6 kilos.

Bread was then omitted and glycosuria stopped, but hyperglycemia continued notwithstanding exercise. October 18, though the weight had fallen to 12.8 kilos, the increasing hyperglycemia and beginning of acidosis gave warning that the diabetes had reached a dangerous stage. The experiment was therefore ended and an attempt made to save the animal. It proved impossible to restore tolerance for any living diet, and death finally occurred February 25, 1917, at a weight of 8 kilos.

This experiment had been planned for testing the practical benefits of exercise in clinical treatment. It showed (1) that exercise favors carbohydrate assimilation even over long periods, especially in mild diabetes; (2) that it loses its effectiveness not with time but with in-

creasing severity of the diabetes; (3) that it can atone to some extent for indiscretions in diet and weight but cannot safely be used as a substitute for dietary restriction, and the attempt to force the diet to the utmost and burn up the surplus calories with exercise ends finally in disaster.

CONCLUSIONS.

1. The influence of exercise on carbohydrate assimilation was traced from the normal through various stages of impairment. A rise of plasma sugar, presumably representing increased transportation, ordinarily accompanies exercise in the normal animal, and the assimilation for test doses of glucose is increased. In mild diabetes, when there is a tendency to abnormal hyperglycemia from defective assimilation of carbohydrate, exercise markedly diminishes the hyperglycemia and glycosuria and facilitates utilization. This power of exercise to improve assimilation applies to the glucose formed from protein diets or body stores as well as from preformed carbohydrate. It does not depend upon the febrile temperatures which attend heavy exercise in dogs, for equal results were obtained in human patients without important elevations of temperature. It is not lost with long usage but becomes less as the diabetes becomes more severe. At a certain advanced stage exercise is unable to modify hyperglycemia or glycosuria. Beyond this, in the extreme forms of diabetes in partially depancreatized animals and in totally depancreatized animals the extra mobilization of sugar by exercise results in an actual increase of glycosuria and of the D:N ratio.

2. With regard to diabetic theory, these results seem to indicate: (a) That the increased metabolism of exercise does not impose an added strain upon the internal pancreatic function; (b) that the combustion of food materials through the increased muscular metabolism and mass resulting from exercise is a definite relief to the internal pancreatic function as compared with the accumulation of such materials through inactivity; (c) that the internal pancreatic secretion is nevertheless an indispensable intermediary in such combustion, and that exercise merely enables the muscles to make more active use of such quantity of this secretion as is available to them but cannot compensate when this quantity falls below the necessary minimum.

3. For purposes of practical treatment the combustion of food by exercise is preferable to its deposit in the body, but exercise cannot replace dietary restriction or permanently atone for excessive diets. The fundamental value of exercise is probably as a form of undernutrition. The combustion of calories by exercise, however, is not as beneficial as omitting them from the diet, and loses its potency at a stage when dietetic undernutrition is still effective. Impairment of sugar utilization by exercise occurs only in the extreme stages of diabetes, but in human patients the nervous and systemic influences must also be considered. With any important degree of undernutrition heavy exercise involves undesirable fatigue and strain, but light exercise aids health. Rest is necessary in the severest cases. ' In the clinical application, therefore, dependence for the actual control of the diabetes is placed upon diet, and exercise is limited to the requirements of comfort and hygiene. The thorough dietetic treatment thus involves two changes from former practice: on the one hand heavy exercise as advocated by the earlier clinicians for burning up surplus sugar is discouraged; on the other hand the hygienic benefits of lighter exercise are made available to many patients to whom exercise was formerly forbidden.

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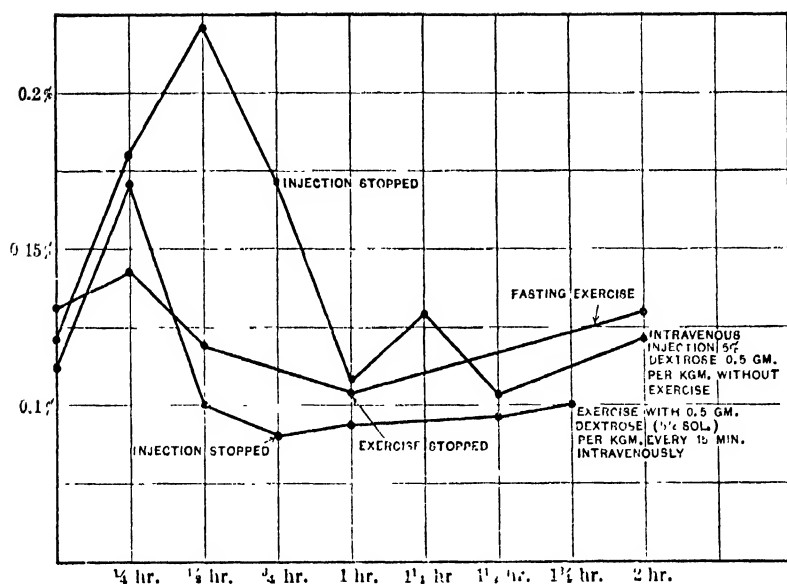
CHART I.—Dog C3–54. Normal. Weight 19 kgm.
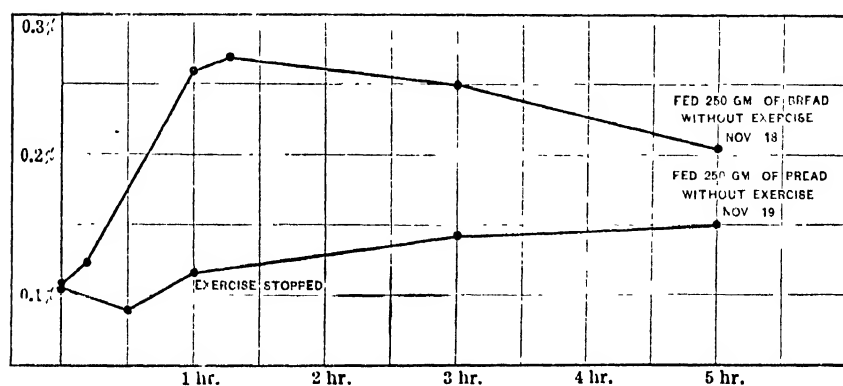
CHART II.—Dog B2–63. Weight 30 kgm. Mild diabetes.
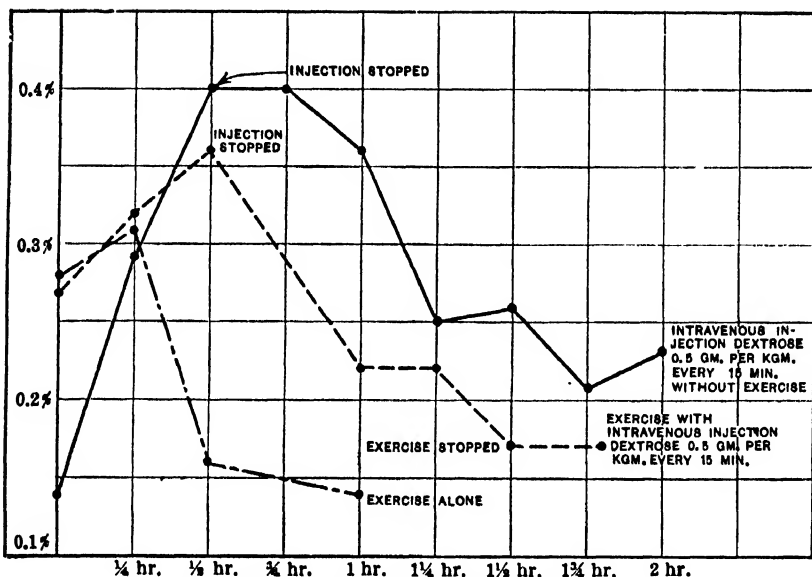
CHART III.—Dog B2-79. Mildly diabetic.
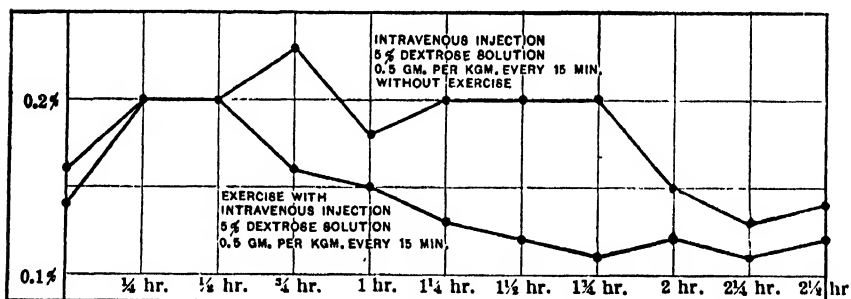
CHART IV.—Dog B2-88. Moderate diabetes.
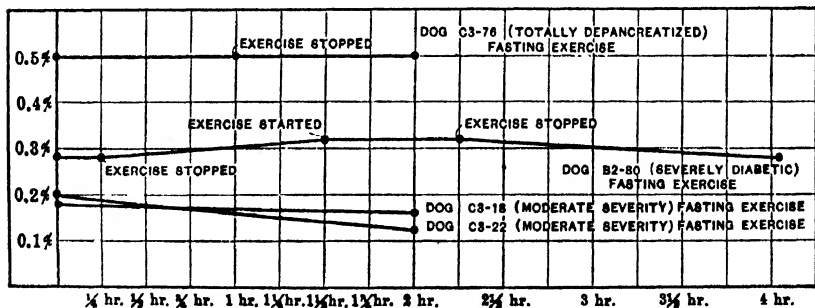
CHART V.—Exercise in severely diabetic dogs.

EXPERIMENTAL STUDIES IN DIABETES.

SERIES II. THE INTERNAL PANCREATIC FUNCTION IN RELATION TO BODY MASS AND METABOLISM.

4. PANCREATIC CACHEXIA.

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Notice of the subject of cachexia is necessary in connection with this series of papers, particularly with a view to the possible criticism that undernutrition treatment represents merely a suppression of glycosuria by cachexia, which may mask the diabetic condition without benefiting it. For the sake of unity other aspects of cachexia must be included in the same paper. They add further to the similarity between clinical and experimental diabetes and thus strengthen the application of results gained with the one form to the other. The marked and rapidly fatal cachexia of totally depancreatized dogs, and their deficiency of wound-healing power and liability to infection, have been known from the time of Minkowski. Attention was previously given[1] to Hedon's "diabetes without glycosuria" and to cachectic disorders in partially depancreatized dogs with or without glycosuria. The present observations will be discussed under the following headings:

Acute deaths following operation.
Subacute and chronic pancreatic intoxication.
Diabetic asthenia.
"Gangrene."
Collapse and prostration as related to glycosuria.
Prolonged cachexia.

ACUTE DEATHS FOLLOWING OPERATION.

Deaths from infection occur sometimes, evidently due in part to diabetic lowering of resistance. Clean surgery prevents peritonitis

[1] Allen, F. M.: Studies Concerning Glycosuria and Diabetes, 1913, chapter x.

even after total pancreatectomy, until after some days pus may burrow through all the suture lines. Young diabetic animals are as subject to distemper as human patients to tuberculosis.

The highest non-infectious mortality occurs in senile, obese dogs, generally within one to three days after operation, with an extreme and progressive prostration, generally without glycosuria or acidosis. Dogs in advanced senility without obesity ordinarily pass through such operations safely. Likewise young obese dogs generally survive the operation easily, though when the obesity is extreme and there is much trauma of the pancreas remnant death may occur from the so-called "fat necrosis." At corresponding intervals after operation, examination of the animals which survive with those which succumb shows no perceptible difference in the changes either in and about the pancreas remnant or in the fat elsewhere. It can only be supposed that senility lowers the resistance to the toxic action of the pancreatic juice.

Pregnant animals, regardless of their nutritive state, are known to be highly susceptible to pancreas operations, which are generally followed by abortion and death within one to several days, though various other abdominal operations may be borne safely. As the fatal result is sometimes so early, it is difficult to ascribe it always to septicemia or other secondary consequences of the abortion, and a special susceptibility of the pregnant animal to pancreatic poisoning seems probable.

Prophylaxis of the fatalities in both obese and pregnant dogs has been attempted by two methods, namely, by preliminary intra-peritoneal injections of watery extract of dog pancreas and by pre-paratory removal of small portions of pancreas. The benefit of either method was doubtful. A very old and fat dog sometimes dies from the quick removal of a small fraction of the pancreas. During the period of injections or successive operations the animal sometimes loses considerable weight, and a diminished susceptibility may be thus explainable. A few successful results were obtained with preparation by either method, but a few were also obtained without preparation.

The above statements are based upon a large series of observations, most of which are included in preceding or subsequent papers. For the sake of brevity it has seemed best to omit individual protocols in this place.

SUBACUTE AND CHRONIC PANCREATIC INTOXICATION.

After the usual partial pancreatectomy, which leaves a remnant communicating with its duct, dogs are sometimes more or less depressed and unwell for several days, and lose weight rapidly, partly on account of indigestion and diarrhea. Swelling and inflammation are generally evident in the pancreas remnant during this period. Still greater decline of weight and strength are commonly found when a portion of pancreas is left isolated from its duct, either in the peritoneal cavity or under the skin. The disturbances are generally transitory, continuing a few days or weeks according to circumstances. They are evidently due to toxic products of pancreatic tissue which is inflamed or undergoing absorption. The early wasting and other symptoms are therefore not attributable entirely to the diabetes, and care should be used in the interpretation of any metabolic observations during this period. Later under suitable conditions the animals are able to digest reasonably well, gain weight and behave normally except for their diabetic tendency, and then are reliable test objects for metabolic study.

DIABETIC ASTHENIA.

If a totally depancreatized, a partially depancreatized and a phlorizinized dog lose equal quantities of sugar in a given time, the totally depancreatized animal will ordinarily show weakness and depression out of all proportion to the other two, and will die while the others are still strong. Even if the totally depancreatized animal appears lively a test with running reveals his lack of strength. The asthenia of partially depancreatized dogs generally appears late in the period of advanced emaciation. Exceptional partially depancreatized animals show early asthenia and rapid progress to death, almost equal to the consequences of total pancreatectomy. These results seem to be classifiable under three headings: (*a*) Small size of remnant; (*b*) inflammation in remnant; (*c*) unknown causes.

(*a*) Small size of remnant.

Occasional dogs with very small pancreas remnants are strong and long-lived. An example previously published[2] was dog 19, which had

[2] (Ibid.), pp. 480, 962.

only about one fifty-fourth of the pancreas, and died in an operation two and a half months later, having been very vigorous for a severely diabetic animal in the meantime.   As a rule, animals with very small remnants suffer not only from poor digestion but also from a more rapid decline than can be accounted for by indigestion.   Some instances of exceptionally rapid decline are the following:

DOG B2–64.

Male, bulldog mongrel; brindle; age five or six years; medium flesh; weight, 14.7 kilos.   May 25, 1914, removal of pancreatic tissue weighing 26.5 gm.   Remnant about main duct estimated at 1.35 gm. ($\frac{1}{20}$ to $\frac{1}{21}$).   Glycosuria was checked by intermittent fasting and meat feeding, so as to be absent part of the time and never above 1 per cent. (2.6 to 12.8 gm. daily output).   Nevertheless the asthenia was out of proportion to the loss of weight, and exceeded that of other fasting partially depancreatized diabetic dogs.   June 15, at a weight of 9.6 kilos, 0.2 gm. of pancreatic tissue was removed for examination. June 20 the dog was sacrificed when moribund.   The pancreas remnant weighed 1.1 gm., and the autopsy otherwise was negative. Microscopically, the tissue on June 15 was normal except for marked vacuolation of islands, while that on June 20 showed recent inflammation in addition.

Though life was not extremely short, the noticeable feature was the disproportionate cachexia in comparison with the glycosuria.

DOG D4–75.

Male; bull terrier mongrel; brindle, aged four or five years; slightly thin; weight, 13 kilos.   January 19, 1917, removal of pancreatic tissue weighing 35.3 gm.   Remnant about main duct estimated at 2 gm. ($\frac{1}{18}$ to $\frac{1}{15}$).   The rapidity of decline and the deficiency of healing of the abdominal wound resembled total pancreatectomy.   On January 26 the dog was very weak and was killed.   The general autopsy was negative grossly and microscopically.   The pancreas remnant was swollen and weighed 5.9 gm.   Microscopically there was extreme inflammation; the acini stained irregularly and were in various stages of involution and repair; islands were scarce and small and showed early vacuolation.

In this animal the two factors, small size and inflammation of remnant, were evidently combined.

Cats are more subject than dogs to this form of cachexia. They become diabetic with remnants between one-fourth and one-fifth of the pancreas, and when the remnant is much smaller there is cachexia resembling that of total pancreatectomy.

CAT A1–80.

Female; maltese; young adult; slightly thin; weight, $2\frac{3}{4}$ kilo. November 13, 1913, removal of pancreatic tissue weighing 5.4 gm. Remnant about main duct estimated at 0.4 gm. ($\frac{1}{14}$ to $\frac{1}{15}$). The animal showed characteristic depression, weakness and poor wound healing, without peritoneal infection, and refused all food. The urine record was as follows:

Date.	Volume.	Glucose.
	cc.	per cent
November 13	60	0
14	100	2.8
15	90	3.8
16	40	3.3
17	80	6.0
18	80	2.7
19	65	2.8
20	50	2.9
21	35	3.1
22	40	4.0

Death occurred November 22, at a weight of 1800 gm. The urine recorded is that found in the bladder at autopsy. In contrast to the emaciation of trunk and limbs there was a surprising quantity of omental and peritoneal fat. The pancreas remnant was swollen and hard and weighed 1.5 gm. Microscopically it showed much inflammation and fibrosis, together with the usual diabetic vacuolation of islands. The other viscera were normal grossly and microscopically, except for slight fattiness of the liver and the Armanni vacuolation of renal tubules.

CAT A1–81.

Female; young adult; gray striped; good condition; weight, 3 kilos. November 14, 1913, removal of pancreatic tissue weighing 8.3 gm. Remnant about main duct estimated at 0.4 gm. ($\frac{1}{21}$ to $\frac{1}{22}$). The animal ate about 25 gm. meat on November 16 and 17. Otherwise the clinical course was characterized by refusal of food, profound asthenia and rapid decline, with the following urine record:

Date.	Volume.	Glucose.
	cc.	*per cent*
November 14	20	0
15	75	1.5
16	50	3.6
17	50	7.1
18	80	2.5
19	70	1.6
20	60	0.7

The cat was found dying on the morning of November 21 and was killed. The weight was 2 kilos. The liver was very large and fatty, the other viscera were normal. The pancreas remnant, normal in appearance and consistency, weighed 0.6 gm. Microscopically it showed fibrosis, chiefly of interlobular type. The acini were in all stages of fulness, emptiness and involution. Islands were almost impossible to find in a series of eight slides containing four sections each. One definite one was found in the last stages of hydropic exhaustion. The others were apparently reduced to small clumps of alpha cells, the identity of which could sometimes be suspected but not clearly demonstrated amid the inflammation. The process of functional exhaustion was thus exceptionally rapid.

(*b*) Inflammation in remnant.

DOG E5–12.

Male; Dalmatian; aged three years; good condition; weight, 16.25 kilos. March 23, 1917, removal of pancreatic tissue weighing 40.5 gm. The attempt was made to leave a remnant small enough to permit diabetes, yet extending the whole length of the uncinate process.

The remnant left consisted of the duct and bloodvessels, with a narrow band of accompanying parenchyma. The dog showed slight appetite on the first two days, but was not fed. There was marked and increasing prostration like that following total pancreatectomy, with the following urine record:

Date.	Volume	Glucose.
	cc	*per cent*
March 23	370	Slight
24	146	1.7
25	810	2.9
26	470	1.8
27	468	3.8
28	248	3.3
29	328	2.4

The dog was found dying on the morning of March 30. There was intense inflammation of the pancreas remnant and distinct congestion and edema of the hypophysis, otherwise nothing unusual in the autopsy.

DOGS C3–83 AND D4–16.

Dog C3–83 became diabetic after removal of five-sixths of the pancreas on April 20, 1916, and dog D4–16 after removal of $\frac{11}{12}$ of the pancreas on the following July 14. The two animals were used for parallel experiments, involving similar glycosuria and other conditions. The contrast in the strength of the two was striking throughout. Dog D4–16 died August 23 while the other dog was still vigorous. The protocols are not given in detail because nothing could prove that some extraneous factor was not the cause of the difference. The number of such observations furnishes evidence that the peculiarity is not accidental. One noteworthy feature in the present instance is the hypertrophy of the pancreas remnant of Dog D4–16, which was estimated at 4.4 gm. at operation and found to weigh 8.2 gm. at autopsy. It thus weighed actually more than the remnant of dog C3-83, which did not hypertrophy. It consisted of normal appearing acinar tissue without fibrosis, with varying numbers of islands. The digestive power should therefore have been as good as in dog C3–83. On the other hand, other studies indicate that the hypertrophy is an indication of preceding inflammation.

(*c*) Unknown causes.

DOG E5–26.

Male; Newfoundland; black, aged three years; good condition; weight, 24.1 kilos.   April 25, 1917, removal of pancreatic tissue weighing 33.7 gm.   Remnant about main duct estimated at 5.7 gm. $(\frac{1}{7})$. Glycosuria was absent until bread was fed on April 27 and thereafter was heavy.   The dog maintained good appetite and cheerfulness, and behaved normally except for surprising weakness, such that he could stand or walk only with great effort.   On May 6 the diet was changed to 200 gm. suet, which was fed daily until May 14, after which lung was added.   The glycosuria rapidly diminished and was absent after May 11, and strength was regained at a corresponding rate, even on the protein starvation.   The animal remained strong thereafter in the aglycosuric condition.   Such asthenia in early diabetes and gain of strength on complete fasting are sufficiently familiar in human diabetes, but are rare in dogs.   No special cause was found in the later history or autopsy of this animal.

CAT B2–06.

Male; yellow and white; old; very fat; weight, 4.2 kilos.   May 12, 1914, removal of pancreatic tissue weighing 8.4 gm.   Remnant about main duct estimated at 2.9 gm. $(\frac{1}{4})$.   The animal ate well and showed heavy glycosuria on milk and meat feeding.   At the same time the weakness was marked and progressive, and death occurred June 1 at a weight of 2.8 kilos.   The bladder at autopsy contained 55 cc. urine, with 1.8 per cent. sugar.   Except for the very fatty liver, nothing noteworthy was found in the viscera.   The pancreas remnant, normal in appearance and consistency, weighed 1.9 gm.   Microscopically there was no inflammation or fibrosis except a trifle at the margins.   The acini were normal except for minimal zymogen content, which may explain the small weight of the remnant.   Islands were present in fair number but were maximally vacuolated.

REMARKS.

Asthenia is known as a prominent symptom of diabetes, which occurs in the late stages of diabetes in all patients and animals, along with emaciation and other severe disturbances.   It occurs much

earlier in some patients, most totally depancreatized dogs and in exceptional partially depancreatized animals. Patients generally regain strength under treatment in proportion to the diet they become able to assimilate, but a few continue to complain of weakness even when free from glycosuria and hyperglycemia on diets which are theoretically adequate. Possible causes of asthenia may be considered as follows:

(a) *Loss of Body Fat.*—In patients asthenia may be out of proportion to the state of apparent nutrition, may even be present with obesity, and varies between individuals independently of the body weight. Depancreatized dogs, especially if obese before operation, may die while still rich in fat, and at all stages are weaker than normal dogs with a corresponding degree of emaciation.

(b) *Loss of Sugar or Nitrogen, or Both.*—The above experiments did not include nitrogen analyses, but there is little doubt that phlorizinized dogs, unless they happen to die from acidosis, may far surpass totally depancreatized dogs in strength and in duration of life, while losing even greater quantities of both glucose and nitrogen. Patients sometimes exhibit asthenia when their carbohydrate and nitrogen balance are still fairly favorable, and the great majority of them gain strength under fasting and reduced diet[3] even though they lose sugar and nitrogen during the process.

(c) *Severity of Diabetes.*—If the severity of diabetes be judged by the D:N ratio and respiratory quotient, it is known that totally depancreatized dogs do not necessarily exhibit the maximal ratio, especially during fasting; also partially depancreatized dogs in the later stages of their diabetes may show maximal ratios (and presumably corresponding respiratory quotients) while retaining greater strength and liveliness and living much longer than totally depancreatized animals. If severity be judged by lack of island tissue, a resemblance exists between animals which have had the pancreas completely removed, or in which the remnant is of minimal size or damaged by inflammation, or in which all the beta cells have been lost by hydropic degeneration, as in some of the examples given above. But many animals retain fair strength and live for a number of weeks or months after their

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[3] Ergometric tests in patients were made by J. R. Williams, Arch. Int. Med., 1917, xx, 399–408.

beta cells are thus lost, while many patients and occasional animals (*cf.* dog E5-26 above) exhibit marked asthenia while the diabetes is still early and mild. In human cases the asthenia is not even a sign of intrinsic severity for it is complained of in many cases which are benign and easily controllable throughout. Cases with asthenia out of proportion to the apparent nutrition after cessation of glycosuria are generally grave, but not more so than many others in which the strength is longer retained.

(*d*) *Nervous Influence.*—A neurotic element may be assumed in many patients, whose nervous systems are specially susceptible to the depression of diabetes. Some differences in this respect may be supposed to exist also between animals, but a more important cause is indicated by the fact that in them the asthenia so often progresses to early death.

(*e*) *Indigestion.*—Totally depancreatized dogs and those with very small remnants naturally suffer from impaired digestion and diarrhea. Whether the cause be purely an enzymatic or partly an endocrine deficiency, pancreas feeding in numerous trials in this series has never been able to restore anything like normal digestion. But the differences in strength are found also in fasting animals, and the asthenia of human patients seldom has any relation to digestion. ·

(*f*) *Intoxication.*—In some animals absorption of pancreatic poisons may play a part, but this is obviously not the case after total pancreatectomy, and it is seldom to be suspected as a cause of the asthenia of human patients. Acidosis is accompanied by a similar and very profound weakness in both patients and animals, but this factor was excluded in the animal experiments, and the type of asthenia in question is often found in patients without acidosis. Toxemia of intestinal or unknown origin is assumed by some writers in connection with human diabetes, but there is no reason why totally or partially depancreatized dogs, especially if fasting, should suffer from intestinal intoxication, and the assumption of unknown toxins merely cloaks ignorance and confuses the problem unless some proof of their existence is produced.

(*g*) *Endocrine Deficiency.*—Asthenia is a fairly common symptom of endocrine deficiency, even in cases which involve no impairment of nutrition in the ordinary sense. Addison's disease and some hypo-

physeal disorders are familiar examples. The most probable theory of diabetic asthenia, aside from the part which is explainable by impaired food assimilation, is that it is an effect of the lack of pancreatic hormone either upon the body cells directly or upon other organs of internal secretion. It would thus be easily comprehensible that this effect might differ somewhat with differences of individual constitution.

"GANGRENE."

A similarity has long been recognized between the gangrene of human cases and the deficiency of wound-healing in totally depancreatized dogs. The dry gangrene which is the commonest clinical form arises always on a basis of arteriosclerosis, and as dogs seem to be not subject to arteriosclerosis, they do not exhibit this form of gangrene. The other form of so-called gangrene, consisting in superficial infections and ulcers which spread instead of healing, occurs in only a small minority of either dogs or patients, and about as frequently in one as in the other. Mention was made in Series I[4] of dog B2–56, which showed unusually rapid downward progress and cachexia. With this there was a development of trophic ulcers as marked as in any human case. One of these perforated into the right elbow-joint and gave rise to a large abscess in the axilla. Other examples are the following:

DOG B2–11.

Female; mongrel; black and white, aged two years; good condition; weight, 6 kilos. November 20, 1913, removal of pancreatic tissue weighing 16.9 gm. Remnant about main duct estimated at 0.6 gm. ($\frac{1}{29}$). Though the remnant was small and the feces accordingly somewhat bulky, the dog showed no more than the usual glycosuria and other symptoms on meat diet. Early in December something caused a trivial abrasion of the right ankle joint. By December 8 the whole leg was hot and swollen, without any definite sinus leading from the point of abrasion. Incisions released considerable pus but did not stop the spread of the infection. Death occurred with pneumonia December 11, at a weight of 3 kilos.

[4] Jour. Exp. Med., 1920, xxxi, 560 and 594.

DOG B2–46.

Male; mongrel; yellow, aged four years; thin but strong; weight, 23.25 kilos. March 26, 1914, removal of pancreatic tissue weighing 32.5 gm. Remnant about main duct estimated at 5.9 gm. ($\frac{1}{6}$ to $\frac{1}{7}$). Glycosuria remained absent until bread feeding was begun on March 30, and was heavy thereafter (up to 6 per cent. in above 2000 c.c. of urine). The asthenia was more marked than in any of the dogs mentioned above as examples of asthenia, and the susceptibility to "trophic" or progressive ulceration equalled or exceeded that of human cases. Death occurred by hemorrhage on April 13.

Other examples will be omitted for the sake of brevity. Certain facts are available concerning the cause of the abnormal liability to infection and necrosis: (1) It is not due to simple malnutrition, for carbuncles and intractable ulcers occur in obese diabetics who are apparently in nutritive equilibrium, and are much commoner among diabetics than among equally undernourished patients with other diseases; (2) it is known that control of the glycosuria and hyperglycemia is the most important means of preventing or curing these conditions, and for this reason speculation has attributed their cause to direct injury of the excess of sugar by osmotic or other action or to the favoring influence of sugar upon bacterial growth. A previous attempt[5] failed to produce any of the supposed consequences of sugar excess by long-continued injections of glucose or other sugars. In more recent experiments subcutaneous injections of 0.1 gm. phlorizin daily have been found to keep the blood sugar of totally depancreatized dogs at a low level, but their impaired healing power, resistance to infection and other disorders have not been in the least benefited. Furthermore the immense majority of partially depancreatized dogs exhibit healing and resisting power practically identical with the normal, even when their hyperglycemia and glycosuria exceed those of totally depancreatized dogs in both degree and duration. The mere loss of sugar and nitrogen from the body is evidently not responsible, for totally phlorizinized dogs bear operations reasonably well. The factor of dryness of the tissues is also thus controlled, for some phlorizinized or partially depancreatized dogs evidently

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[5] Allen, F. M.: 'Studies Concerning Glycosuria and Diabetes, 1913, chapter iii.

undergo greater desiccation than the average totally depancreatized dog. (3) The actual endocrine deficiency, in addition to any of the above factors, must be invoked here as in the case of asthenia. The almost uniform difference between totally and partially depancreatized animals is thus explainable, and the special incidence in individual animals or patients may be accounted for by constitutional variations in resistance and the occurrence of predisposing injuries.

COLLAPSE AND PROSTRATION AS RELATED TO GLYCOSURIA.

Examples were previously given[6] of the prevention of glycosuria by weakness or prostration, and numerous others might be added from the present series. Dog D4–76 underwent operation on January 19, 1917, and died January 22 from necrosis of most of the pancreas remnant. Here the diabetes should have approached that of a totally depancreatized animal, but the principal symptom was prevented by intoxication. The instance is mentioned because of the similarity to some human cases of fulminating pancreatitis. In Series I, reference was made to animals in which glycosuria was stopped and the progress of diabetes halted by distemper or other causes of anorexia and emaciation. Such animals sometimes later enjoyed excellent health and a high food tolerance in consequence of their spontaneous undernutrition treatment. When the diabetes has become established in a sufficiently severe form, it cannot be halted by fasting or any other known means. In the writer's experience totally depancreatized dogs as well as the partially depancreatized ones with the severest diabetes ordinarily die after continuous fasting or any intercurrent illness or accident with heavy glycosuria present to the end. In the monograph (1913) already mentioned, references were made to the literature concerning absence of glycosuria in totally depancreatized animals after such extreme procedures as removal of the liver or adrenals, or high section of the spinal cord. Any therapeutic significance of such results can be imagined only on the basis of two fallacies, one the confusion between glycosuria and diabetes and the other the indefensible doctrine of diabetes as a pure overproduction of sugar. If it be believed that an animal is diabetic because it cannot

[6] (Ibid.), p. 492.

assimilate carbohydrate without the internal secretion of the pancreas, obviously the removal of some other organ cannot restore the lost power. Likewise the cessation of glycosuria in occasional totally depancreatized animals in the last hours of life has no therapeutic significance. The very fact of total absence of the pancreas renders such animals unfit for therapeutic experiments unless it is a question of some extract or other agent which can replace the pancreatic function. The results of the undernutrition treatment of patients and partially depancreatized animals are significant for both theory and practice because it can be shown that they do not represent a mere suppression of glycosuria by cachexia, but accomplish an actual raising or preservation of the power of food assimilation and permit a long or indefinite continuance of life.

PROLONGED CACHEXIA.

The following are rare instances in which diabetes seemed to be superseded by some unknown cachectic condition, producing results different from those of simple undernutrition. Cats seem to be more liable than dogs to this abnormality.

CAT A1–91.

Male; gray striped; middle aged; slightly thin; weight 3.1 kilos. January 13, 1914, removal of pancreatic tissue weighing 5.8 gm. Remnant about main duct estimated at 1.8 gm. (about $\frac{1}{4}$). There was glycosuria on milk and meat diet and none on meat alone. The appetite and health seemed excellent. The glycosuria was allowed to continue and increase up to 5 per cent. in 455 c.c. urine on February 6. February 7, at a weight of 3.2 kilos, fasting was begun. The glycosuria immediately fell to traces, rose to a moderate reaction on February 10, then fell and was absent after February 12. February 16, at a weight of 2.6 kilos, feeding was begun with 20 gm. cooked beef-lung, increasing gradually until on February 23, 140 gm. lung and a live mouse were fed. The weight meanwhile had been falling rapidly to 1.8 kilos; the cat retained appetite and appeared well except for remarkable weakness and weariness. Lung, suet and raw pancreas were then fed as abundantly as possible. The digestion

seemed rather poor, as indicated by bulky formed feces, but the cachexia was more advanced and progressive than is seen in plain fasting. Death occurred February 26, and no cause could be found at autopsy. The microscopic examination of liver, kidneys, adrenals and thyroid was negative. The pancreas remnant, normal in appearance and consistency, weighed 1.8 gm. It was fully normal microscopically; the acini were well filled with zymogen; islands were abundant and free from vacuolation.

CAT A1–94.

Female, adult; white and black; thin; weight, 2 kilos. January 21, 1914, removal of pancreatic tissue weighing 3.7 gm. Remnant about main duct estimated at 0.8 gm. ($\frac{1}{5}$ to $\frac{1}{6}$). The animal refused food except for a trifle of milk, and the highest glycosuria was 0.5 per cent. in 25 c.c. urine. Death occurred on Januray 25, with urine sugar-free, at a weight of 1.6 kilos. Autopsy showed no cause. The pancreas remnant weighed 0.6 gm., and was normal in acini and islands.

The history of cat A1–95 was closely similar.

Some atypical results of this character caused the writer formerly[7] to conclude wrongly that cats are unsuitable for diabetic experiments.

CAT A1–96.

Male; gray and white; large, strong adult; excellent condition; weight, 3.4 kilos. February 9, 1914, removal of pancreatic tissue weighing 5.6 gm. Remnant about main duct estimated at 1.4 gm. ($\frac{1}{5}$). Glycosuria was produced at first only by milk. It was present March 1 to 23 on meat diet, then ceased spontaneously. With addition of milk it was present March 30 to April 9. On this date two tiny fragments of pancreas, with an aggregate weight of 0.15 gm., were removed for microscopic study. Then on fasting, glycosuria was absent after April 11. A diet of beef-lung or liver was begun with 50 gm. on April 13, increased rapidly to 300 gm. and then *ad libitum*. The cat meanwhile rapidly lost weight and strength without

[7] (Ibid.), p. 502.

glycosuria, and died April 26, at a weight of 1.9 kilos. The autopsy revealed no gross or microscopic cause of the condition. The pancreas remnant weighed 2.25 gm. Microscopically the tissue removed April 9 was normal except for marked vacuolation of islands. At autopsy there was some interlobular fibrosis; the acini were nearly empty but otherwise normal; islands were scarce in some sections, abundant in others, with most of their cells normal but a few showing slight persisting vacuolation.

In several dogs glycosuria has been unexpectedly absent or has ceased for unknown cause after it has been present for a longer or shorter time. These instances of cachexia have confirmed the previous conclusions[8] that the duration of life is generally far shorter than in cases in which diabetes develops in typical form. The most marked example of prolonged cachexia was the following:

DOG D4–70.

Male; mongrel; white and black; shaggy; aged four years; medium nutrition; weight, 15.5 kilos. January 10, 1917, removal of pancreatic tissue weighing 31.6 gm. Remnant about main duct estimated at 2.6 gm. (about $\frac{1}{13}$). There was no glycosuria until bread and milk was fed on January 13, causing a slight reaction which disappeared on the same diet. Beginning January 18 a diet of bread and 200 gm. glucose maintained glycosuria until January 23, when it ceased. Thereafter the dog ate the same full pan of bread, with 300 gm. glucose, 200 gm. lung and 100 gm. suet without glycosuria. Beginning January 27, analyses of urine and feces were performed on a diet of 200 gm. lung, 250 gm. bread and 100 gm. suet, which showed only a moderate deficiency of food absorption; also a genuinely high tolerance was demonstrated by an intravenous glucose test and a series of blood sugar analyses, but all these records were lost. February 10, 0.5 gm. phlorizin suspended in oil was injected subcutaneously, and heavy glycosuria continued until February 15, indicating a full ability to form and excrete sugar under this stimulus. The weight had mostly remained low, between 13 and 14 kilos, but rose by April 4 to 16.4 kilos. At this weight the feeding of bread and soup

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[8] (Ibid.), p 500.

ad libitum with 100 gm. glucose brought on glycosuria, which ceased promptly when the diet was changed to 400 gm. lung and 200 gm. suet. By May 4 the dog was still lively and happy, with enormous appetite, but was showing hair loss and unsteadiness of legs. Because the symptoms resembled those of fat intoxication (produced in normal dogs by diets too high in fat and low in protein) the diet was changed on May 22 to lung and bread, with milk occasionally, and bonemeal to supply salts. On June 12 and July 27 small portions of pancreas were removed for microscopic examination without giving rise to glycosuria. The pancreas remnant appeared normal grossly and

microscopically. The body weight fell as low as 10.3 kilos, notwithstanding edema, but rose later to 12.5 kilos. Death occurred, apparently from simple weakness, on September 27. The pancreas remnant, slightly firm and nodular, was in free communication with its duct, and weighed 2.9 gm. Microscopically it showed slight fibrosis, interacinar as well as interlobular, but the acini were large and well filled and the islands abundant and normal. Gross and microscopic examination of the other viscera revealed no abnormalities to account for the condition.

The above photograph was taken on July 5. The dog unfortunately was not photographed when normal, but was a rather hand-

some animal with long thick hair.   The hair loss is seen on the head and ears, trunk, limbs, and the naked end of the tail visible between the legs.   Edema without evidences of nephritis is occasionally seen in cachectic dogs, even without pancreas operations.   It was greater here in the hind than in the front limbs, and was not like myxedema, but consisted in an accumulation of clear fluid which flowed freely from a needle puncture.   The dog remained in excellent spirits throughout.   Signs of sexual activity were completely lacking, and the animal was somewhat silly in mentality, as J. H. Pratt has stated[9] concerning young dogs with pancreatic atrophy.

CONCLUSIONS.

1. In addition to some acute deaths from unknown causes shortly after partial pancreatectomy, examples were given of diabetic asthenia and diabetic gangrene in animals, which add another detail to the resemblance between clinical and experimental diabetes.   Reasons were given for considering both the asthenia and the gangrene as due not to simple malnutrition, hyperglycemia, glycosuria or other causes, but rather to the specific endocrine deficiency.

2. Examples were also given in which the diabetes of dogs and cats was replaced by a fatal cachexia of unknown nature.   It is not certain that the condition in all these instances was the same.   Malnutrition due to impaired digestion or intestinal absorption was probably one factor, but the results seem to differ in some respects from simple undernutrition, and may possibly bear some relation to the metabolic alterations in states of prostration which suppress glycosuria even after total pancreatectomy.

3. It is highly important to distinguish the mere suppression of glycosuria by some injury, poison or cachexia from genuine control or improvement of the diabetes.   It is thus impossible to use totally depancreatized dogs for any therapeutic experiments, unless for testing some agency which is supposed to replace the internal pancreatic function.   The results of the undernutrition treatment of animals and patients are distinguished from mere cachectic suppression of glycosuria by the fact that the power of food assimilation is demonstrably strengthened, the general health improved and life lengthened instead of shortened.

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[9] Personal communication.   Also Jour. Am. Med. Assn., 1912, lix, 322–325.

EXPERIMENTAL STUDIES IN DIABETES.

Series II. The Internal Pancreatic Function in Relation to Body Mass and Metabolism.

7. The Influence of Cold.

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According to some earlier literature (1), cold causes hyperglycemia and glycosuria in both warm- and cold-blooded animals, and increases the glycosuria of diabetic dogs. The use of cold and shivering to drive out glycogen from phlorizinized animals was familiarized by Lusk. A single mild test of cold environment in a human patient gave a negative result (2).

A few words may be devoted to the theory of the subject. In a totally phlorizinized dog, it is obvious that a release of carbohydrate which cannot be utilized will result in a temporary rise of glycosuria and of the D : N ratio. A similar mobilization of stored carbohydrate, together with a possible diuretic action of cold, might produce a similar result in a totally depancreatized animal. The extra heat required to maintain body temperature would supposedly be furnished by fat, and there is no reason to expect any increased output of sugar derived from protein, either in the sense of any appreciable increase of protein decomposition or any genuine alteration of the D : N ratio. In a partially depancreatized animal, the increased carbohydrate mobilization or increased total metabolism may impose an additional burden upon the pancreas remnant, and any lasting increase of glycosuria must be interpreted in the sense of a true aggravation of the diabetes. At the same time complicating factors come into play. If the animal fasts, the extra sugar loss and fat combustion impose a sharper undernutrition treatment in a cold than in a warm environment. Likewise a diet which is adequate in warmth becomes under-

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nutrition in the cold.   On the other hand an increase of diet to meet the increased requirement complicates the problem still further. Shivering is a form of muscular exercise, and may possibly have a metabolic effect opposed to the effect of cold *per se*.

Normal dogs can live without artifical heat in the ordinary winter weather of New York if merely sheltered, but their metabolism evidently is considerably higher than that of dogs kept in warm rooms. The first experiments were therefore planned to determine whether there is any practical difference in the ease of producing diabetes in dogs kept in outdoor cages in winter and in others kept in a room specially warmed to a high summer temperature.   This method was considered better for the purpose than the plan of using severely diabetic dogs and following the variations in their glycosuria, because it was important to distinguish a tendency to the production of mere glycosuria and hyperglycemia (such as cold may excite even in normal animals, which are certainly not diabetic from this cause) from a tendency to the production of actual diabetes.   If cold has any diabetogenic action, equivalent to the removal of a small fraction of a gram of pancreatic tissue, this action should be demonstrable in such tests.

Twenty-five dogs were used for this investigation, with removal of such portions of pancreas as were known to produce a close approach to diabetes.[1]   These experiments included fasting and fixed diets, also single and repeated operations, the latter as usual removing successive bits of tissue till diabetes resulted.   Comparisons were made between dogs kept in the warmth and others kept in the cold, and also in the same animals by sudden changes from one environment to the other. The animals chosen ranged from small short-haired dogs which were highly sensitive to the cold and might sometimes be unduly depressed by it, to large woolly dogs which scarcely shivered in the winter weather.   The general technic of such experiments is sufficiently clear from the preceding papers, so that brevity may be served by omitting protocols.   In two instances the change from warmth to cold seemed responsible for a definite but transitory glycosuria. Otherwise the results were negative, and the conclusion was estab-

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[1] All operations were performed under ether anesthesia.

lished positively that there is no demonstrable difference in the amount of pancreatic tissue that must be removed to produce diabetes in dogs in warm or cold environment. An effect of cold upon the islands of Langerhans was also not observable.

Although cold has no diabetogenic influence whatever in the sense of this test, it thus merely conforms to the rule that the most powerful functional influences avail little in comparison with the smallest fraction of a gram of healthy pancreas tissue. As agencies which are negative in this respect sometimes appreciably influence the course of an existing diabetes, some experiments concerning hyperglycemia and glycosuria were performed upon dogs with various grades of carbohydrate tolerance. For this purpose the animals' cages were transferred from a comfortably warm room to a refrigerator room kept at approximately freezing temperature. The observations are arranged in a series chiefly according to descending assimilative capacity.

Dog C3–36.

TIME	PLASMA SUGAR	REMARKS
	per cent	
August 6		
10:00 a.m........	0 128	At summer temperature
3:40 p.m	0 116	Immediately after this bleeding, transferred to ice room
5:50 p.m	0 098	
August 7		
10:00 a.m........	0 113	
4:00 p.m........	0 100	Immediately after this bleeding, transferred from ice room to summer temperature
6:00 p.m	0 095	
9:25 p.m........	0 105	

The normal dog C3–36, weighing 17 kilos, was placed in the ice room after the bleeding at 3:40 p.m. on August 6, and left there until after the bleeding at 4 p.m. on August 7. The usual diet of bread and soup was fed each evening after the final blood sample was taken. The dog was in excellent condition and powerfully muscled, but very short haired, and shivered continuously in the cold. The plasma sugar seemed to be affected very slightly if at all. At any rate, no elevation by cold was observed.

Dog B2-00, mentioned several times in previous papers, in August, 1915, was close to the verge of diabetes, but had been free from glycosuria on fixed bread diet for several months.   Exercise had proved able to modify the blood sugar and the glucose assimilation considerably, and an experiment with cold was therefore performed under similar conditions.   The diet was fed each evening after the last blood test.   There was no glycosuria.   The plasma sugar was slightly lower at the low temperature.

TIME	PLASMA SUGAR	REMARKS
	per cent	
August 5		
11:30 a.m.........	0.111	
2:30 p.m........	0.117	At summer temperature
August 6		
9:30 a.m.........	0.110	
3:30 p.m........	0.098	Immediately after this bleeding, transferred to ice room
5:45 p.m........	0.085	
August 7		
9:30 a.m.........	0.098	
3:30 p.m........	0.107	Immediately after this bleeding, transferred from ice room to summer temperature
6:00 p.m........	0.125	
9:30 p.m........	0.112	

Dog B2–01.

PLASMA SUGAR			TIME
September 21, Cold	September 29, Control	October 6, Exercise	
per cent	*per cent*	*per cent*	
0.116	0.106	0.105	Blood before feeding
0.192	0.256	0.147	1 hour after feeding
0.106	0.147	0.148	3 hours after feeding
0 131	0.103		6 hours after feeding

Dog B2–01, likewise near the verge of diabetes, received 56 grams Merck anhydrous glucose (4 grams per kilo) in 30 per cent solution by stomach tube on three days, respectively in the cold, in warmth at rest, and with treadmill running. The control day showed the highest plasma sugar curve, while both cold and exercise seemed to depress it.

Dog B2-02.

PLASMA SUGAR			URINE SUGAR		TIME
July 20, Control	September 23, Cold	October 15, Exercise	July 20, Control	September 23, Cold	
per cent	*per cent*	*per cent*	*per cent*	*per cent*	
0.144	0.118	0.114	0	0	Before feeding
0.270	0.228	0.111	4.50 (13 cc.)	Slight	1 hour after feeding
0.149	0.115	0.100	1 85 (84 cc.)	0	3 hours after feeding
0.112	0.100		0	0	6 hours after feeding

Dog B2-02 was known to have very mild latent diabetes, but was continuously free from glycosuria on bread diet. Tests similar to those of dog B2-01 were carried out with the giving of 30.5 grams Merck glucose (3 grams per kilo) by stomach tube. Cold seemed to reduce hyperglycemia and glycosuria as compared with the control day. On the exercise day there was by far the lowest plasma sugar and no glycosuria.

Dog D4-29.

PLASMA SUGAR

October 4, Control	October 9, Cold	October 10, Control	
per cent	*per cent*	*per cent*	
0 093	0.120	0.147	Blood before feeding
0.307	0.357	0 256	1 hour after feeding
0.307	0.455	0 202	2 hours after feeding
0.322	0.400	0 235	3 hours after feeding
0.307	0.416	0.222	4 hours after feeding
0.313	0.364	0.250	5 hours after feeding
	0.322	0 206	8 hours after feeding
	0.125	0 200	13 hours after feeding

Dog. D4-29.—Female; mongrel; age 2 years; good condition; weight 11.5 kilos. September 28, 1916, removal of pancreatic tissue weighing 26.9 grams. Remnant about main duct estimated at 3.8 grams (⅛). The removal of 0.65 gram additional tissue on October 16 was necessary to produce diabetes. In the interval the tests shown in the table were performed, with the feeding of 200 grams bread and 150 grams glucose on each occasion (together with 200 grams talcum powder as a precaution against diarrhea). On October 9 the dog was transferred to the cold room immediately after feeding. The plasma sugar curve ran noticeably higher on this day than on the control days, but yet was lower at the end of 13 hours, as if more of the available carbohydrate had either been excreted or consumed by shivering. An accident prevented comparison of the glycosuria.

Dog B2- 86.—The history of this animal was given in paper 2 of the preceding series (3). The animal had been on the verge of diabetes with an unusually large pancreas remnant, and enormous quantities of bread and glucose had been necessary to keep up glycosuria, but by July 21 this was disappearing and the appetite was failing. Therefore the expedient was adopted of raising the animal's metabolism to the highest level possible, by exercising him on the treadmill to the limit of his strength daily, and keeping him in the ice room all the rest of the time, in the endeavor to bring on diabetes either by stimulation of appetite or by any direct influence upon the pancreas. July 22 was occupied in this manner. July 23, exercise was deferred until a feeding experiment with 400 grams bread and 500 grams glucose could be carried out in the ice room in comparison with the one at summer temperature on July 21.

TIME	PLASMA SUGAR	
	July 21, at summer temperature	July 23, at freezing temperature
	per cent	*per cent*
Before feeding	0 124	0 100
2 hours after.	0 147	0 128
7 hours after.............	0 141	0.141

The lower sugar curve on July 23 may have been partly the result of the preceding day's exercise, but at least augured failure for the undertaking. The program of combined exercise and cold was continued daily, with addition of as much as 600 grams of glucose to the bread diet, up to the time of the second operation on August 7. The strong animal merely thrived on the program, and such diabetic tendency as had seemed to be present disappeared.

TIME	URINE		REMARKS
	Volume	Glucose	
	cc.	*per cent*	
July 28			
2:30 p.m........		0	Fed 50 grams bread
5:00 p.m........	38	0.28	
July 29			
2:30 p.m........		0	Fed 50 grams bread and placed in ice room
5:00 p.m........	47	0	Returned to summer temperature
July 30			
10:00 a.m........		0	Fed 50 grams bread and placed in ice room
5:00 p.m........	214	Trace	Returned to summer temperature
July 31			
10:00 a.m........		0	Fed 50 grams bread
5:00 p.m........	275	0.4	

Dog B2–71.—June 3, 1914, at a normal weight of 14.7 kilos, nine-tenths of the pancreas were removed. In July, 1915 the dog was still in a state of moderate diabetes, kept sugar-free on meat diet, at a weight of 12.5 kilos, and was used for exercise and other experiments. Some tests were then performed with feeding 50 grams of bread, following only the urine without blood analyses. The regular lung diet was given each evening after completion of the test.

In this experiment there was distinctly greater glycosuria on July 28 and 31 in warm summer weather than on July 29 and 30 at freezing temperature.

Dog C3–00.—Female; fox terrier mongrel; white and brown; age 5 years; good condition; weight 4.5 kilos. May 6, 1915, removal of pancreatic tissue weighing 11 grams. (Remnant about main duct estimated at 1.25 grams ($\frac{1}{10}$). As sometimes happens in small dogs, glycosuria was absent on full meat diet even with this small pancreas remnant. On May 15 a change to bread and soup promptly brought heavy glycosuria, which ceased with a return to meat diet on May 21. A fixed diet of 750 grams lung was then given daily, but was not always eaten completely. After continuous absence of glycosuria, the dog was transferred to the ice room on May 29, and in the following 24 hours excreted 0.45 per cent sugar in 375 cc. urine. Glycosuria then continued absent on the same diet as before, until on June 7 the dog was removed from the ice room. At summer temperature a return to bread and soup diet produced immediate heavy glycosuria. Accordingly, in this experiment cold failed to maintain glycosuria on meat diet in a dog which was demonstrably diabetic as proved by glycosuria on bread diet.

In August the same dog was tested with the aid of plasma sugar analyses on a regular diet of 500 grams lung and 50 grams suet. There was no glycosuria.

TIME	PLASMA SUGAR	REMARKS
	per cent	
August 10		
10:45 a.m.........	0.135	Immediately after this bleeding, transferred to ice room
12:00 noon........	0.122	
4:45 p.m.........	0.141	
August 15		
12:30 p.m........	0.182	
August 16		
12:45 p.m........	0.098	
4:45 p.m........	0.112	Immediately after this bleeding, transferred from ice room to summer temperature
August 18		
12:00 noon........	0.167	Summer temperature
5:25 p.m........	0.143	

Dog B2–88.

PLASMA SUGAR					TIME
November 15, Control	November 18, Control	November 20, Cold	November 23, Cold	November 26, Exercise	
per cent	*per cent*	*per cent*	*per cent*	*per cent*	
0.128	0 189	0 131	0.122	0.162	Blood before feeding
	0 250	0 222		0 263	½ hour after feeding
	0.257	0 233		0.189	2 hours after feeding
0.286			0.271		4 hours after feeding

Dog B2–88, with mild diabetes, received test meals of 200 grams bread and 100 grams beef lung. The plasma sugar curve seemed to vary chiefly according to the initial figure, without much influence of any of the special measures employed. Thus, both figures on November 15 are a trifle higher than on November 23 in the refrigerator. Also the differences between November 18, 20 and 26 seemed to be governed chiefly by the level on which the plasma sugar started. Cold at least did not elevate the plasma sugar.

Dog B2–58.—Female; mongrel; yellow; age 3 years; good condition; weight 11.8 kilos. Fasting was begun May 4, 1914, and by May 21 the weight was reduced to 9.1 kilos. It was thus possible to remove ⅓⅓ of the pancreas on this date with only a moderate degree of diabetes resulting. After various tests, the tolerance was spared by a low protein diet, which could be gradually increased by October 20 to 1 kilo of beef lung daily, without glycosuria. The weight gradually rose by December 24 to 12.3 kilos, on which date the first glycosuria appeared. About the same time the dog began to leave part of the diet uneaten, and the weight thus fluctuated and in general fell. The partial checking of glycosuria, and its daily variations, are thus accounted for. Beginning January 6, 1915, the dog's cage was kept outdoors, in order to test the effect of cold upon the diabetes either directly, through stimulation of appetite or in any other way. The dog had time to become acclimated because the weather was fairly mild at first, but after January 20 it turned decidedly colder, and there were several days with considerable snow and ice. As the dog was short-haired, she was shivering practically continuously while outdoors, but passed through even the coldest weather without actual impairment of health. The rectal temperature remained normal. Contrary to expectation, the amount of food eaten was not appreciably different in the cold environment. After February 12 the glycosuria was checked by fasting. The experiment seems to indicate a slight increase of glycosuria by cold, but the influence certainly was not great.

Dog B2-58.

DATE	WEIGHT	URINE	
		Volume	Glucose
	kgm.	*cc.*	*per cent*
December 24		320	1.0
25		585	0.5
26	12.8	470	Faint
27		385	0.2
28		950	Faint
29		355	0
30	12.7	384	0
January 1		360	0
2		500	0
3		632	0
4		650	Faint
5		480	0
6	12.7	330	0
7	12.4	502	0
	today placed in cage outdoors		
8		431	0
9		307	0
10		575	0
11	11.1	713	0
12		766	0
13	11.2	646	0
14		954	0
15	10.9	698	0
16		382	0
17		Not collected	
18	11.1	1849	0
19		988	0.6
20	11.3	995	0
21		965	0.7
22	11.1	781	0.9
23	11.2	609	0.5
24		740	0.9
25	10.9	700	Faint
26	11.1	749	0
27	11 0	900	Very faint
28	11.3	731	1.5
29	11.5	630	1.0
30	11.5	156	2.0
31		820	1.1

Dog B2–58—Concluded.

DATE	WEIGHT	URINE	
		Volume	Glucose
	kgm.	*cc.*	*per cent*
February 1	11 3	1036	1 0
2	11.3	1300	0 8
3	11 0	950	2.0
4	11 1	785	1.8
5	11.4	1157	2.0
6	11 8	605	2.5
	Today moved into	animal room	
7		650	1.0
8	11 5	973	0 9
9	11 5	649	0 4
10	11.7	733	0 7
11	11 5	815	Faint
12		600	Faint

Dog B2–89.—This female mongrel, weighing 13 kilos, underwent partial pancreatectomy on April 12, 1915, leaving a remnant of $\frac{1}{17}$. Under regulated diets, the condition by the end of June was such that glycosuria was absent at a weight of 10.3 kilos on a diet of 1 kilo of lung, but substitution of 250 grams lung by 50 grams bread (making 750 grams lung and 50 grams bread) caused glycosuria of ・ 0.75 per cent in 462 cc. urine on June 30 and 0.71 per cent in 542 cc. urine on July 1. On the latter date the diet of 1 kilo of lung without bread was resumed, and glycosuria immediately ceased (4). On July 2 the dog was transferred to the ice room, in order to test whether the influence of cold would amount to as much as the above difference between carbohydrate and protein. The dog was left in the ice room until July 26. Traces of glycosuria were present on most days during this time, but only twice reached titratable amounts (0.33 per cent on July 14, 0.25 per cent on July 21). A single day of exercise on July 22 abolished the glycosuria, which returned on July 24. It was present also on July 25 and 26, and then was continuously absent during a control period up to August 5 at summer temperature. As the weight fell to 9.7 kilos during the period in the refrigerator, the experiment seems to indicate a slight increase of diabetic tendency due to cold. The difference due to temperature, however, was evidently less than the difference between the preformed carbohydrate of 50 grams of bread and its approximate equivalent of potential carbohydrate in protein. The tolerance had fallen somewhat, for the giving of 50 grams of bread on August 15 resulted in a glycosuria of 1.4 per cent.

Glycosuria remained absent on the lung diet to September 17. On that day at 9:30 a.m. the plasma sugar was 0.143 per cent, the rectal temperature 101.4°F.

The dog was then placed in the ice room fasting. At 4 p.m. the plasma sugar was 0.151 per cent, the rectal temperature 100.7°F. The usual lung diet was then fed and the dog left in the ice room. The next morning there was a glycosuria of 0.42 per cent in 567 cc. urine, and the plasma sugar at 9:30 a.m. was 0.164 per cent. The urine of the next 24 hours was 492 cc., with 1.9 per cent sugar. At 9:30 a.m. on September 19 the rectal temperature was 101.5°F. Glycosuria then ceased abruptly, as though the reserve of extra carbohydrate had been exhausted. At 9:30 a.m. on September 20 the plasma sugar was 0.146 per cent. The dog was then transferred to the warm animal room, and at 9:30 a.m. on September 21 the plasma sugar was 0.133 per cent, the rectal temperature 101.7°F.

During the following days the weather turned colder. The night of September 23–24 was particularly sharp, and the door of the animal room blew open, so that the room was cold and the dogs all shivering. This dog was one of five potentially diabetic animals (out of about twenty) which had been kept sugar-free on regulated diets and which showed sudden glycosuria on this night.

Subsequently, at a body weight of 15 kilos and correspondingly reduced tolerance, a comparison was made in this dog of the feeding of 1 kilo of meat in a warm room and in the ice room.

TIME	PLASMA SUGAR	
	November 26, (warm room)	December 3, (ice room)
	per cent	*per cent*
Before feeding	0.200	0 208
2 hours after feeding......................	0 218	0 278
5 hours after feeding..........	0 208	0 313
Glycosuria for the period.....................	0	4 08 grams

The later observations showed a decided influence of cold for the production of hyperglycemia and glycosuria. It is possible that this effect became greater as the diabetes became more severe.

Dog B2–79.

PLASMA SUGAR		URINE SUGAR		TIME
November 23, Warm	November 30, Cold	November 23, Warm	November 30, Cold	
per cent	*per cent*	*per cent*	*per cent*	
0.133	0.250	0	Faint	Blood before feeding
0.213	0 416	Faint	5 00	1 hour after feeding
0.159	0.525	Faint	5.90	4 hours after feeding
0.189	0.435	0	3.53	6 hours after feeding

Dog B2–79 was an animal which had long been kept in a stage of diabetes such that there was marked hyperglycemia and occasional glycosuria on a diet of 1 kilo of beef lung, the condition being held in check by fasting when necessary. The above record shows a strong contrast in hyperglycemia and glycosuria on days spent in a warm room and in the ice room respectively. The rise of blood sugar after eating the usual kilo of lung was somewhat similar on the two days, but the entire curve was on a much higher level in the cold environment. It was not established that this difference was due entirely to the temperature, because no comparison was made of the blood sugar before and after moving into the refrigerator on November 30. Therefore on December 2 the plasma sugar was determined fasting at 11:40 a.m. and found to be 0.164 per cent. The dog was then moved into the cold room, and at 5:40 p.m. the plasma sugar (still fasting) was found to be 0.185 per cent.

Likewise dog C3–19, weighing 13.8 kilos, was partially depancreatized on June 23, 1915, leaving a remnant of $\frac{1}{11}$ to $\frac{1}{12}$. In August at a weight of 11 kilos, the degree of diabetes was such that a diet of 500 grams lung was slightly in excess of the tolerance. On August 7 the fasting plasma sugar at 10:30 a.m. was 0.156 per cent and at 4:30 p.m., 0.120 per cent. The dog, still fasting, was then moved to the ice room, where the plasma sugar was found to be 0.167 per cent at 7 p.m. and 0.133 per cent at 9:45 p.m. Here a falling blood sugar due to fasting was evidently raised by cold, and then continued to fall slightly.

DISCUSSION.

The elevation of blood sugar by cold is so generally accepted as a truism that it was a surprise to encounter instances in which the sugar was little changed or actually lower in a cold environment. It was not feasible to extend the investigation further into the physiological reaction to cold. Supposedly the blood sugar is governed by two factors, namely the mobilization and the disposal of carbohydrate. It is conceivable that in a perfectly smoothly working reaction the two may exactly balance. Under some conditions shivering may perhaps reduce the blood sugar like other forms of muscular activity. Under other conditions hyperglycemia may occur, particularly when the cold stimulus is sufficiently violent, as for example in the case of plunging into ice water. Here the stimulation is so powerful, sometimes to a pathological degree, that a correspondingly excessive sugar discharge may be expected, and in the most extreme cases possibly the utilization of sugar suffers somewhat. Hyperglycemia and glycosuria may be more readily produced when the utilization

of sugar is specifically impaired, as in the more severe grades of diabetes. In a similar way exercise sometimes raises the blood sugar instead of lowering it.

Violent or pathological stimulation by cold was not applicable in experiments designed to produce diabetes, because the animals' health would suffer and ill health would be the surest way to spoil the result and prevent diabetes. A more powerful temporary discharge of sugar might occur, but it would cease as soon as the immediate store was exhausted. Such discharge and cessation was actually seen in certain of these experiments, without any lasting diabetogenic effect. As usual, clear thinking requires a distinction between diabetes, which is deficiency of the pancreatic function, and mere glycosuria. The mere excessive discharge of sugar from the glycogen depots is not diabetes, for the power of utilization may remain unimpaired. This has been abundantly proved, for example, in such a condition as epinephrin glycosuria (5). It is also not diabetes if the utilization of sugar is depressed by any extraneous mechanism, such as the chilling of the muscles or their nervous supply, but only if the impairment of utilization is due to impairment of the pancreatic function. If the glycosuria produced by cold is regarded as a diabetes, cold must be a very powerful diabetogenic agent to cause even a temporary diabetes in a normal animal, for it must thus temporarily paralyze about nine-tenths of the function of the dog's pancreas. Trial of this agent in animals depancreatized almost to the point of diabetes proves that it possesses no such power; and as the effect in these animals is not greatly different from that in normal animals, cold evidently does not act by direct depression of the pancreatic function. Its indirect influence upon diabetes through increasing metabolism is a much more delicate point to demonstrate, and the question is answered only somewhat doubtfully in the affirmative by some of the above experiments.

CONCLUSIONS.

1. Cold environment, such as did not lower the rectal temperature to any important extent, in some instances failed to affect the plasma sugar of dogs or slightly lowered it, but in the majority of experiments

produced hyperglycemia and sometimes glycosuria. These were produced more easily and in higher degree in proportion as the power of sugar utilization was impaired, i.e., as the diabetes was more severe.

2. The power to produce glycosuria is to be distinguished from the power to produce diabetes. There is no demonstrable difference in the proportion of pancreatic tissue that must be removed to produce diabetes in dogs in warm or cold environment, and it was proved by successive operations upon the same animals that the influence of cold is not equivalent to the removal of the smallest fraction of a gram of pancreatic tissue. In animals already diabetic, the course of the diabetes in a few instances seemed to be influenced slightly for the worse, so as perhaps to warrant the conclusion that cold imposes an increased burden upon the pancreatic function by increasing metabolism. But the slightness of this influence is emphasized by control experiments; for example, it amounts to less than the difference between the preformed carbohydrate of 50 grams of bread and the approximate equivalent of potential carbohydrate in protein.

3. The impression that diabetic patients do worse in cold weather is probably explainable by the discomfort of chilliness when they are undernourished, the tendency to take more food, and sometimes by the limitation of exercise. These may be important sometimes from a practical standpoint, but any direct influence of climate upon diabetes must be very slight if it exists.

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EXPERIMENTAL STUDIES IN DIABETES.

Series II. The Internal Pancreatic Function in Relation to Body Mass and Metabolism.

8. The Influence of Extremes of Age upon the Production of Diabetes.

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An influence upon the production of diabetes may conceivably be expected from the conditions of extreme age or youth.

Senility.—The relation of senility to experimental diabetes may have interest from at least three standpoints: *a*, The total metabolism is known to be slightly lowered in old age; *b*, diabetes in elderly patients is generally characterized by a mild and prolonged course; *c*, the increasing incidence of diabetes with the advance of age suggests the possibility of functional or organic impairment of the pancreas.

Toothless decrepit senility is familiar in dogs, and there is reason to expect as great metabolic changes as in aged human beings. Obesity is also common in such animals, so that a number of them had to be included in a previous paper (1). A comparison of the relation of pancreas weight to body weight was made in fourteen dogs, which showed extreme senility together with an average nutritive state.

From a comparison of table I with a similar study of normal adult dogs (2), it may be inferred that there is no gross change, in particular no atrophy of the pancreas accompanying senility.

The susceptibility of obese senile dogs to diabetes could seldom be determined, owing to the sudden death to which they are subject following pancreas operations, as previously mentioned (3). Such operations were performed without accidents in senile dogs without obesity, and these were used for diabetic experiments in the same way as younger dogs. No experiments were performed to determine the

precise proportion of pancreas that must be removed to produce diabetes, but the incidental observations were sufficient to exclude any marked differences from average adult dogs. The senile animals were somewhat weaker and more subject to loss of appetite and cachexia. Otherwise their diabetes ran a course indistinguishable from that of younger animals. The experimental answers may therefore be stated in the following form, corresponding to the questions raised above.

TABLE I.

Relation of Pancreas Weight to Body Weight in Senile Dogs.

NUMBER	BODY WEIGHT	PANCREAS WEIGHT	PANCREAS PER KILOGRAM BODY WEIGHT
	kgm.	*grams*	*grams*
1	4 0	15.0	3.75
2	4.4	7.0	1.59
3	5.8	9.5	1.64
4	8.8	23.1	2.62
5	11.3	19.6	1.73
6	11.3	24 4	2.16
7	11.5	25 6	2.23
8	11.7	32.1	2.74
9	14.2	53.6	3.78
10	15.0	33 6	2.24
11	16 0	20.2	1.26
12	21 3	46 4	2.18
13	21.8	39.6	1.81
14	25.1	41.4	1 65

a. The lowered metabolism characteristic of senility does not render dogs less susceptible to diabetes from pancreatic resection. As the reduction of metabolism with age is slight, and the method of judging susceptibility by the size of the pancreas remnant with which diabetes occurs is a crude one according to former observations, too much stress need not be laid upon these negative findings.

b. A more decisive observation is that diabetes does not run any slower course in senile animals, but follows the same rapid progress which is generally characteristic of diabetes in dogs.

c. If there were any anatomic or functional deterioration of the pancreas with age, a remnant of a given size might be expected to be less efficient in preventing diabetes than in younger animals. An

increased susceptibility to diabetes in this sense is excluded by the observations. The microscopic study, as reported in subsequent papers, showed no visible abnormalities resulting from simple senility. Pancreatitis is much rarer in dogs than in human beings, and is apparently due to causes independent of age. Also there is no such arteriosclerosis in senile dogs as in man, and it can by no means be said that a dog "is as old as his arteries." Also, as previously mentioned (2), the pancreas remnant of a senile dog may possess as great power of hypertrophy as that of any younger animal.

Numerous glucose tolerance tests have been performed indiscriminately upon old and young adult dogs, and no indications of alteration of assimilation with age have been found.

In general these observations, made upon a species practically free from the pancreatic changes to which man is subject with advancing years, indicate that the rising incidence and special characteristics of diabetes in older persons are due to the changes in question and not to senility *per se*.

Youth.—The outstanding feature of experimental interest is the characteristically rapid and fatal course of diabetes in children. This has often been attributed hypothetically to their high metabolism, which imposes a heavier burden upon the pancreatic function. There is also good evidence that this rapid downward progress indicates a susceptibility of the islands of Langerhans to rapid destruction by hydropic degeneration. As there are occasional cases of acute and severe diabetes in the aged and of mild and prolonged diabetes in children, there is no absolute distinction on the basis of either the level of metabolism or the island changes.

Diabetes is also less frequent in children than in older persons. In seeking possible reasons, it might be imagined that the youthful pancreas is larger in proportion to the body, that it is anatomically richer in islands or that these have stronger functional power, or that the capacity for regeneration after injuries is greater. According to work previously reviewed (4), especially that of Bensley, the pancreas at birth probably contains as many islands as the adult organ, but during early life there is probably a loss followed by a gradual new formation of islands. Observations on the gross relations of the pancreas in puppies are contained in tables II to VI. They indicate,

TABLE II.

Litter of Black and Tan Mongrel Pups.   Weight of Mother, 17 Kilos.

NUMBER	SEX	AGE	BODY WEIGHT	PANCREAS WEIGHT	PANCREAS WEIGHT PER KILOGRAM
			kgm.	*gram*	*grams*
1	Male	Newborn	0.3	0 6	2 0
2	Male	Newborn	0.3	0.6	2 0
3	Female	Newborn	0 2	0 4	2.0
4	Female	Newborn	0 3	0.5	1 67
5	Male	Newborn	0 3	0.6	2.0
6	Female	Newborn	0 3	0 7	2 33
7	Male	Newborn	0 4	0.8	2 0
8	Female	Newborn	0 3	0 9	3.0
9	Male	Newborn	0 3	0 6	2 0
10	Male	Newborn	0 3	0.8	2.66
11	Female	Newborn	0 3	0 6	2.0

TABLE III.

Litter of Harrier Mongrel Pups.   Weight of Mother 13 Kilos.

NUMBER	SEX	AGE	BODY WEIGHT	PANCREAS WEIGHT	PANCREAS WEIGHT PER KILOGRAM
			kgm.	*gram*	*grams*
1	Female	1 day	0.2	0.3 .	1 5
2	Male	1 day	0 2	0 3	1.5
3	Male	1 day	0.3	0 6	2 0
4	Male	1 day	0 3	0.4	1.33
5	Male	1 day	0 3	0 7	2 33
6	Male	1 day	0 2	0 7	3 50

TABLE IV.

Litter of Spaniel Mongrel Pups.   Weight of Mother, 16 Kilos.

NUMBER	AGE	BODY WEIGHT	PANCREAS WEIGHT	PANCREAS WEIGHT PER KILOGRAM
		kgm.	*grams*	*grams*
1	Slightly premature	0 2	0.7	3 5
2	Slightly premature	0.2	0.6	3.0
3	Slightly premature	0.3	0.8	2.67
4	Slightly premature	0.3	0.6	2.0
5	2 days	0.3	0.9	3.0
6	3 weeks	0.6	2.1	3.5

in comparison with those on adult dogs (2), that the ratio of pancreas weight to body weight in puppies is not large but rather small in proportion to what might be expected from the small size of the animals; that the tendency to regeneration is often marked but yet not in excess of that often found in adults; and that the tendency to diabetes is at least no greater and often is distinctly less than in adult dogs.

In qualification of this statement, it should be noticed that the observations do not exclude possible alterations of the ratio of pancreas weight to body weight with age. In other words, there is no proof that a puppy having a certain ratio will maintain this same ratio up to adult age. In tables IV and V no great change was noticed in ratio up to 2 or 3 weeks of age, using other pups of the same litter as controls. The ratios varied widely among the different pups

TABLE V.

Litter of Yellow and Brown Mongrel Pups. Weight of Mother, 21.5 Kilos.

NUMBER	AGE	BODY WEIGHT	PANCREAS WEIGHT	PANCREAS WEIGHT PER KILOGRAM
		kgm.	*grams*	*grams*
1	½ week	0 3	1.1	3.67
2	½ week	0.4	1.7	4.25
3	½ week	0.4	1.3	3.25
4	2 weeks	0.6	2.6	4.33
5	2 weeks	0.7	2.8	4 00

in table VI. They were sometimes larger in the smaller breeds, as found for adult dogs (2), but the rule was not uniform. The state of nutrition or rate of growth is probably an important factor. The differences found among animals of the same litter at the same age in tables II and III interfere seriously with studies based on any method of controls.

Partially depancreatized puppies are especially liable to cachexia from respiratory infections or diarrhea, so that diabetes is often thus suppressed and the experiments spoiled. The above statement concerning the relatively slight disposition to diabetes is based on animals which remained vigorous and thriving after operation. It is the more striking in view of the fact that the sugar tolerance of the young is distinctly less than that of adult normal animals (5). A

series of observations was made upon the question whether partial pancreatectomy short of diabetes interferes with the growth or health of puppies, with negative results except for the temporary backset due to the operation, and a possible specific inhibition of development of sexual and other adult characters.   Anything in the nature of a masked diabetes or other fatal metabolic deficiency seemed to be excluded.

TABLE VI.

Relation of Pancreas Weight to Body Weight in Puppies.

NUMBER	AGE	BODY WEIGHT	PANCREAS WEIGHT	PANCREAS WEIGHT PER KILOGRAM
		kgm.	*grams*	*grams*
1	1 week premature	0.1	0 2	2 0
2	1 week premature	0 1	0.2	2.0
3	1 month	0.7	2 2	3.14
4	1 month	1.5	7.1	4.74
5	1½ months	2.1	4.1	1.95
6	1½ months	1.3	2.6	2.00
7	2 months	0 9	3.5	3.89
8	2 months	2 3	5.1	2.22
9	2 months	2 0	5.9	2.95
10	2 months	1.8	5 8 ·	3 22
11	2 months	4 3	8 4	1 95
12	2 months	2 3	7 4	3.21
13	2¼ months	1 8	7 4	4.10
14	2½ months	2 5	5 7	2 28
15	3 months	2 5	12 2	4.89
16	3 months	2.3	5 2	2 26
17	5 months	3.9	13.8	3.54
18	7 months	2.5	11 4	4 55

As explained in a subsequent publication on acidosis, the expectation that puppies might prove more susceptible than adult dogs to diabetic acidosis was entirely disappointed.   Acidosis was absent throughout the reported experiments except where specially mentioned.

As puppies approach adult age, they approach more closely to the adult behavior respecting diabetes.   After about the ninth month they generally react like adults except for susceptibility to distemper. Nevertheless in pup 18 in table VII it is noticeable that diabetes was

TABLE VII.

Partial Pancreatectomies in Puppies.

NUMBER	AGE	BODY WEIGHT	PANCREAS WEIGHT	PANCREAS WEIGHT PER KILOGRAM	WEIGHT OF REMNANT	SIZE OF FRACTION	HYPERTROPHY	REMARKS
	months	kgm.	grams	grams	grams		grams	
1	1	3.0	13.2	4.4	0.8	$\frac{1}{16}-\frac{1}{17}$	0 8-1.02	
2	2	1.9	9.7	5 1	1.3	$\frac{1}{4}-\frac{1}{8}$	1 3-5 7	Transitory glycosuria. Mild diabates produced by removal of additional 1.2 grams
3	2	1.7	8.9	5.2	0.7	$\frac{1}{13}-\frac{1}{14}$	0.7-0 4	Cachexia. Weight 1 kgm. at autopsy. Probably mild diabetes
4	3	2.5	11.0	4 4	0.5	$\frac{1}{22}$	0 5-0 6	Cachexia. Mild diabetes
5	3	2.1	10.8	5.1	3.0	$\frac{1}{4}-\frac{1}{4}$		Diabetes not produced even by circulatory stasis
6	3	3.9	17.8	4.6	5.7	$\frac{1}{3}$		Diabetes not produced even by circulatory stasis
7	3	2.0	16.0	8.0	2.7	$\frac{1}{6}$	2.7-5.3	No diabetes
8	3	2.1	8.3	3.9	0.8	$\frac{1}{10}$		Thin, no glycosuria
9	4	3.7	15.8	4.3	1.5	$\frac{1}{10}$		Assimilated 5 grams of glucose per kilogram
10	4	8.4	27.6	3.3	4.6	$\frac{1}{8}$		Removal of 3.6 grams additional tissue in 6 operations required to bring on diabetes. Remnant at autopsy was then 1.1 grams
11	5	1.9	7.8	4.1	0.6	$\frac{1}{8}$	0.6-1.0	
12	5½	5.3	25.8	4.9	4.4	$\frac{1}{6}$	5.4-8.4	No diabetes
13	6	8.0	22.5	2.8	9.3	$\frac{1}{2}-\frac{1}{3}$		
14	6	3.8	8.5	2.2	1.0	$\frac{1}{8}-\frac{1}{6}$		
15	7	5.9	20.1	3.4	1 7	$\frac{1}{12}$		Peritonitis, no glycosuria
16	7	5.9	15.0	2.54	1.6	$\frac{1}{6}-\frac{1}{10}$		Diabetes stopped by distemper
17	7	3.4	9 3	2.74	1 5	$\frac{1}{6}$		Cachexia, no glycosuria
18	7	4.4	8.9	2.02	1.0	$\frac{1}{10}$		Diabetes, mild, transitory
19	8	7.2	19.8	2 75	1.8	$\frac{1}{11}$		
20	9	5.8	21.5	3.71	1.3	$\frac{1}{17}$		Diabetes prevented by preliminary fasts
21	9	2.7	10.5	3.90	1.0	$\frac{1}{10}$		Peritonitis, no diabetes

TABLE VII—*Concluded.*

NUMBER	AGE	BODY WEIGHT	PANCREAS WEIGHT	PANCREAS WEIGHT PER KILOGRAM	WEIGHT OF REMNANT	SIZE OF FRACTION	HYPERTROPHY	REMARKS
	months	kgm.	grams	grams	grams		grams	
22	9	7.4	24.8	3.36	2.5	$\frac{1}{10}$		Severe diabetes, even during fasting
23	9	7.1	26.2	3.69	3.0	$\frac{1}{9}$	3.0–1.95	Severe diabetes checked by cachexia. Emaciated to 5.3 kgm.
24	9	10.3	30.9	3.00	3.1	$\frac{1}{10}$	Considerable	
25	11	4.1	14.3	3 50	1.1	$\frac{1}{13}$	1.1–2.2	Cachexia, no diabetes

transitory with a pancreas remnant of only $\frac{1}{10}$. Some typical records of younger puppies are the following.[1]

No. G7–49.—Male; mongrel; black and white; age 1 month; well nourished; weight 3 kilos. June 25, 1918, removal of pancreatic tissue weighing 12.4 grams. Remnant about main duct estimated at 0.8 gram ($\frac{1}{16}$ to $\frac{1}{17}$). Following operation there was diarrhea and loss of weight but no glycosuria, though the pup lived on bread and milk. July 4, 0.32 gram additional pancreatic tissue was removed. The condition was still cachexia without glycosuria, up to death on July 11. The pancreas remnant weighed 0.7 gram, and was too badly autolyzed for microscopic study. In the tissue removed on July 4, the acini were normal and well filled; islands were present in normal number and size but showed slight distinct vacuolation. This hydropic change makes it probable that hyperglycemia was present, and possibly only the animal's weakness prevented a frank diabetes. Acidosis was absent as usual.

No. D4–35.—Female; mongrel; age 3 months; brown and white; well nourished; weight 2.5 kilos. November 9, 1916, removal of pancreatic tissue weighing 10.5 grams. Remnant about main duct estimated at 0.5 gram ($\frac{1}{22}$). Glycosuria was brought on by the feeding of 20 grams meat and 20 cc. milk, but ceased readily with fasting. Death occurred from weakness, notwithstanding a low meat diet, on November 20. The pancreas remnant weighed 0.6 gram, and was not examined microscopically. The pup obviously had a potentially severe diabetes, but differed from an adult dog in that glycosuria did not begin spontaneously and was promptly checked by fasting and low diet, with such a very small pancreas remnant.

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[1] All operations were performed under ether anesthesia.

No. D4–34 similarly underwent removal of 8.2 grams pancreatic tissue, leaving a remnant estimated at 0.65 gram ($\frac{1}{15}$ to $\frac{1}{14}$). Glycosuria could readily be checked by fasting, but on full meat diet was as high as 1.2 per cent. Notwithstanding the liveliness following operation and the meat diet, the weight and strength failed rapidly. The animal was killed when moribund on the tenth day after operation, when the plasma sugar was 0.05 per cent. The rapid cachexia was the chief point of difference from an adult dog.

No. G7–25.—Female; Boston terrier; brindle; age 6 weeks; good nutrition; weight 3.8 kilos. June 17, 1918, removal of pancreatic tissue weighing 13.1 grams. Remnant about main duct estimated at 1.1 gram ($\frac{1}{13}$). Glycosuria remained absent, first on beef lung, then on bread and soup diet, then with addition of 50 grams glucose. June 25, at a weight of 3.5 kilos, 1 gram additional pancreatic tissue was removed, the remnant having undergone marked hypertrophy. Glycosuria remained absent on bread and milk diet, though weight was lost. July 4, 0.95 gram additional pancreatic tissue was removed. The animal was lively and ate considerable lung on July 5, then gradually lost appetite and weight while retaining spirits up to death on July 9. Glycosuria remained absent. The plasma sugar on June 17 was 0.143 per cent, on July 8, 0.162 per cent. The pancreas remnant weighed 0.8 gram. In the various specimens of pancreas from June 17 and July 4 and 9, inflammation and fibrosis were limited to the peripheral areas. In the central portions the acini were well filled, sometimes large and irregular. Islands were normal in number and free from vacuolation. Here the persistent absence of diabetes is perhaps attributable to the hypertrophy of the pancreas remnant.

No. E5–40.—Male; mongrel, part Newfoundland; age 3½ months; good nutrition; weight 8.4 kilos. May 14, 1917, removal of pancreatic tissue weighing 23 grams. Remnant about main duct estimated at 4.6 grams ($\frac{1}{6}$). Glycosuria was absent on bread and soup diet, and only transitory with addition of 100 grams glucose.

May 24, additional pancreatic tissue weighing 0.9 gram was removed. There was obvious hypertrophy of the remnant in all three dimensions. Thereafter glycosuria was absent on bread diet, and could be maintained only by increasing additions of glucose, first 50, then 100, then 200 grams daily. The dosage of 200 grams was continued from June 6 to 27 with only traces of glycosuria and with a gain of weight to 9.3 kilos.

June 27, 0.6 gram additional tissue was removed from the pancreas remnant. which again was obviously hypertrophic. Thereafter heavy but transitory glycosuria resulted first from 100 and then from 200 grams glucose added to the bread diet.

July 12, an additional 0.35 gram of pancreatic tissue was removed, the weight then being 9.8 kilos. Glycosuria was then heavy with addition of 200 grams glucose to the bread diet, but ceased July 19.

July 20, an additional 0.4 gram of pancreatic tissue was removed. Glycosuria was then heavy on bread and soup diet, next with addition of glucose up to 200 grams, but was absent after July 29.

August 31, at a weight of 10.6 kilos, glycosuria being still absent on the bread diet with 200 grams glucose, an additional 0.52 gram of pancreatic tissue was removed.   Glycosuria was again transitory.

September 7, an additional 0.3 gram of pancreatic tissue was removed with a similar result, glycosuria ceasing September 18.

September 28, an additional 0.55 gram of pancreatic tissue was removed. Glycosuria was then continuous, first with glucose, then on plain bread and soup, till stopped by a change to meat diet on October 22.   It may be noted that the downward progress was slow rather than rapid, for the glycosuria of many adult dogs after 3 weeks reaches a point where it cannot be stopped by fasting.

The pup did not thrive on carbohydrate-free diet, suffered from indigestion, diarrhea and loss of weight, and died in cachexia November 16 at a weight of 7 kilos.   The blood sugar was low during this period, and there was the usual absence of acidosis.

The pancreas remnant weighed 1.1 gram.   Nothing significant was found in the gross autopsy, or in the microscopic examination of the liver, kidneys, adrenals, thyroid and parathyroids.   Sections from the pancreas at all the operations and at autopsy showed normal parenchyma and absence of hydropic degeneration.   Though the hypertrophy may have gone far toward preventing diabetes, it is evident from the repeated operations and the prolonged carbohydrate excess that this puppy was difficult rather than easy to make diabetic.

No. D4–21.—Male; mongrel; age 7 months; good nutrition; weight 6.7 kilos. September 15, 1916, removal of pancreatic tissue weighing 24 grams.   Remnant about main duct estimated at 2.9 grams ($\frac{1}{8}$).   Glycosuria at first was absent on . moderate quantities of milk, but was heavy on bread feeding September 18.   It was then checked by fasting, and kept absent on a diet of lung, suet and 100 grams bread.   The animal thrived and maintained a weight of about 7 kilos.

December 5, a full bread diet was resumed, and heavy glycosuria returned promptly.   After 4 days this was stopped as before.   The animal grew to adult life on a diet of 400 grams lung, 100 grams suet, and part of the time 100 grams bread or 100 cc. milk.   Yeast was added part of the time, with the idea that it and the milk might supply needed vitamines; also an admixture of bonemeal assured adequate salts.   The animal became plump and strong at a weight of 8.7 kilos, but remained always undeveloped in mentality, in the size of the sexual organs and the absence of any apparent sexual function; urination was performed squatting, and the puppy contour of the body and puppy-like behavior toward other dogs were retained.   Pratt (6) has made similar observations in young dogs with pancreatic atrophy.   .

November 16, 1917, it was found that the addition of 100 grams bread to the diet produced a glycosuria of 1.3 per cent in 820 cc. of urine.   The tolerance was thus evidently lower than before, and thereafter the diet was kept carbohydrate-free (400 to 600 grams of lung and 100 grams of suet).   April 6, 1918, the fasting plasma sugar was found to be 0.250 per cent, indicating that hyperglycemia was now continuous.   The weight at this time was 9.15 kilos.   Traces of glycosuria

began in July, at first intermittently, but by July 30 they were continuous and increasing, so that fasting had to be used. The weight at this time had reached its maximum of 9.5 kilos. The animal appeared not obese but in excellent nutrition.

The diet was then gradually built up to 200 grams lung and suet ad libitum, without glycosuria. This diet continued while the writer was in military service. At a visit on September 16, 1918, the animal was found in excellent strength and spirits, weighing 8.3 kilos, with intense glycosuria and hyperglycemia and heavy nitroprusside reactions in urine and plasma. Notwithstanding the fat-rich diet and acidosis, there was no visible lipemia. The condition was ideal for the development of coma, but under the circumstances there was nothing to do but to kill the animal for autopsy.

The body appeared in excellent condition and retained much fat. Except for a very fatty liver and the puppy-like characters noted above, the gross autopsy was negative. The pancreas remnant weighed 3.1 grams. The practical absence of hypertrophy may be noticed.

Microscopically, the liver was crammed with fat even to the periphery of the lobules. Stains with Best's carmine showed absolutely no glycogen except in occasional leukocytes, which stood out prominently in the capillaries by reason of their stuffing of red granules.

The kidneys were normal except for maximal Armanni vacuolation, as seen in routine Zenker specimens stained with methylene blue and eosin. Best's carmine applied to the alcohol fixed specimens showed only a sparse sprinkling of glyco-gen, so that the vacuoles evidently represented chiefly fat deposit, as demon-strated by fat stains in some other animals but not in this one.

The adrenals showed no more than the average lipoid vacuolation in the cor-tex, and were normal in other respects.

The thyroid and parathyroids were normal, with average colloid content in the former.

The pancreas remnant was normal in structure and fullness of acini and num-ber and size of islands, but a minority of island cells were maximally swollen and vacuolated.

This puppy, in respect to "spontaneous" downward progress and other features, behaved practically like an adult animal, and most of the history actually represented an adult period of life. Many long experiments of this character were begun for the purpose of testing whether a partial pancreatectomy which did not suffice to produce immediate diabetes in a puppy might result in diabetes after a certain stage of growth had been reached. In other words, the problem was whether a damaged pancreas might lag behind in develop-ment so as finally to become inadequate for the demands of a growing

body. The best observations would have been those upon much younger animals, but all these failed for one cause or another. It is evident that in many cases the hypertrophy of the pancreas remnant fully compensates for any bodily growth. It is also very doubtful whether an animal which is actually non-diabetic will become diabetic by simple growth. But when the injury results in such a mild diabetes that there are no symptoms on whatever dietary regime is followed, it is entirely possible that diabetes may develop openly at a much later period under the strain of growth and gain of weight. Such a result is exemplified in this experiment, which covers a rather long period in proportion to a dog's life. Such experimental evidence makes it easily comprehensible that diabetic symptoms may follow either soon or late after an injury of the human pancreas; in particular, that glycosuria may be present immediately after an infection, or may be delayed to a time when there is no plain clinical connection with a long antecedent infection which may have been the real cause.

<div align="center">CONCLUSIONS.</div>

1. An influence of senility upon carbohydrate assimilation or diabetes is conceivable from a quantitative reduction or other alterations of metabolism, or from functional or anatomic changes in the pancreas. The observations upon senile dogs failed to show any departures from the normal in the glucose tolerance, the ratio of pancreas weight to body weight, the microscopic structure of the pancreas, the size of the pancreatic remnant with which diabetes occurs, the capacity of such a remnant for hypertrophy, or the clinical course of the diabetes.

2. An investigation was also made of the possible influence of the elevated metabolism or the pancreatic peculiarities of youth. Previous work had indicated that the glucose tolerance of puppies is less than that of adult dogs. No exact studies were made of the most important point in the microscopic anatomy, namely the richness in islands, but no striking departure from the adult average was noticeable in numerous routine observations. The ratio of pancreas weight to body weight was somewhat irregular, and the question of possible changes in an individual during growth could not be accurately decided, but the general average in puppies did not differ appreciably

from that in adults, particularly with consideration of the small body weight. The remnant left after partial pancreatectomy generally grows considerably in puppies; the hypertrophy on the whole is greater than in adult dogs, but does not surpass what is found in occasional mature or adult animals, and may be slight or absent especially in older puppies. The tendency to diabetes is distinctly less in puppies than in adult dogs, partly on account of weakness and cachexia, partly because of hypertrophy of the pancreas remnant, and perhaps sometimes because of a high functional efficiency of a small remnant. There is no specific tendency to rapidity of downward progress or to diabetic acidosis in puppies.

3. An example is given of long delayed onset of diabetic symptoms on a fixed diet after gain of weight, this gain being largely growth instead of mere obesity. With a still milder diabetic tendency it is readily conceivable that the delay might be longer and might occur on an ordinary diet, also that the diet might in large measure determine the onset of symptoms. In this way it is possible that childhood infections injuring the pancreas may be responsible for some cases of diabetes which make their appearance at a much later period.

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EXPERIMENTAL STUDIES IN DIABETES.

Series II. The Internal Pancreatic Function in Relation to Body Mass and Metabolism.

9. The Influence of Pregnancy upon Experimental Diabetes.

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Diabetes may conceivably be aggravated by the increased metabolism of pregnancy, which involves both additional food assimilation and the formation of considerable new tissue. Unknown toxic or metabolic factors may possibly have an influence. An opposite possibility is suggested especially by the work of Carlson and collaborators (1), namely, that diabetes in the latter part of pregnancy may be prevented by the internal secretory activity of the fetal pancreas. The chief question here is whether internal secretions pass in appreciable quantities through the placenta. In clinical practice pregnancy has long been regarded as decidedly injurious, to such an extent that therapeutic abortion was commonly recommended in women suffering from any serious grade of diabetes. The most comprehensive and recent survey of clinical experience is by Joslin (2), who considers that the supposed injurious effects are largely explained by the higher diet taken. One case of apparent gain of tolerance during pregnancy (3) is inconclusive, partly because the blood sugar rose during gestation and partly because it is uncertain whether the changes observed were due strictly to the pregnancy.

Partially depancreatized animals offer the most accurate obtainable conditions for studying the effect of pregnancy upon the internal pancreatic function. Before proceeding to the principal experiments, it is desirable to mention briefly some controls and unsuccessful attempts relating to this problem.

One control to be thought of is the possible effect of changes in the sexual organs themselves upon the pancreas or its function. This is part of the larger question of the interrelation of the sex glands with the pancreas and diabetes. A few extirpation experiments were performed as follows.[1]

Dog B2–89, a female mongrel aged 4 years, had been close to the verge of diabetes since the first operation on April 12, 1915, so that the removal of 0.25 gram additional pancreatic tissue on March 16, 1916 brought on mild diabetes. By repeated tests up to May 18 it was proved that glycosuria was regularly absent on a measured bread and soup diet, and was present to the amount of about 5 grams daily with the addition of 75 grams glucose. May 18, both ovaries were removed, together with the tubes and part of the uterine horns. A trace of glycosuria followed the operation, and another trace when the regular bread diet was fed. The addition of 75 grams glucose resulted in glycosuria of 12.5 grams one day and 13 grams another day. By May 27 glycosuria was again absent on the plain bread and soup diet and the glycosuria from glucose was no higher than before operation. The absence of any perceptible influence upon the diabetes, other than the transient effects of the trauma, was also confirmed by the animal's later history.

Dog D4–62.—Female; mongrel; long haired; age 6 to 8 years; well nourished; weight 19.2 kilos. December 16, 1916, removal of pancreatic tissue weighing 27.5 grams. Remnant about main duct estimated at 3.2 grams ($\frac{1}{10}$). After fasting, glycosuria was found consistently absent on a diet of 1 kilo of beef lung, and moderate (0.5 to 1 per cent) with addition of 50 grams bread. January 17, 1917, both ovaries were removed. A trace of glycosuria followed the operation, but ceased on the lung diet. With addition of 50 grams bread it was distinctly greater than before (2.8 to 4.8 per cent, in larger urine volumes), but ceased when the bread was omitted on January 26. Thereafter the diabetic tendency was no greater than before the oöphorectomy.

Dog C3–67.—Male; Dalmatian; age 1½ years; in excellent nutrition; weight 17.6 kilos. March 14, 1916, castration, and removal of pancreatic tissue weighing 28.5 grams. Remnant about main duct estimated at 2.6 grams ($\frac{1}{14}$ to $\frac{1}{15}$). The dog was used for a few tests (especially feeding of pure fat or talcum powder, as described elsewhere) which had no influence upon the glycosuria. Otherwise no food was given, but heavy glycosuria continued until death occurred from weakness on April 1, at a weight of 11 kilos. The pancreas remnant, weighing 3.1 grams, was normal except for the usual vacuolation of island cells. The bladder urine contained 0.61 per cent of sugar, and the autopsy otherwise was negative. Such irrepressible glycosuria with a pancreas remnant of this size is so unusual that the possibility of an aggravating influence of the castration was suggested.

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[1] All operations were performed under ether anesthesia.

Dog D4–63.—Male; mongrel; long haired; age 3 years; medium nutrition; weight 15.25 kilos. After removal of pancreatic tissue on February 14 and March 7, 1917, glycosuria was maintained almost continuously on bread and soup diet with addition of 200 grams glucose to March 23, after which it was absent. Such a duration of glycosuria is evidence that an animal is very close to diabetes, which will be produced by the removal of a trifle more pancreatic tissue. The actual absence of diabetes was confirmed by blood sugar tests, the plasma sugar being 0.087 per cent before feeding and 0.145 per cent at the highest point afterward, and down to 0.122 per cent by evening even on the bread and glucose diet. March 28, castration was performed. Glycosuria remained absent on bread diet to April 2, and thereafter also with addition of 200 grams glucose. The attempts to induce diabetes by diet were continued through May, with merely the usual slight gain in tolerance. The removal of 0.5 gram pancreatic tissue on May 8 proved inadequate, but mild diabetes followed the removal of 0.65 gram additional on June 1.

This single experiment with dog D4–63 probably suffices to prove that removal of the testes has no influence for or against the production of diabetes. Aggravation of an existing diabetes, which is a separate question, was not produced by removal of the ovaries in dogs B2–89 and D4–62. The apparent aggravation after removal of the testes in dog C3–67 may have been purely accidental, and any specific influence remains improbable. Circumstances prevented further experiments of this sort.

Numerous attempts were made to determine the size of pancreas remnant with which diabetes occurs in pregnant animals, with uniform failure on account of abortion or death. Efforts to immunize by one or several preliminary subcutaneous or intraperitoneal injections of aqueous extract of fresh sterile dog pancreas gave no protection. Trials were made with removal of five-sixths to nine-tenths of the pancreas in single operations, performed as quickly and easily as possible so as to be over in half an hour or less, but the animals always became unwell and aborted within a few days or at most a week. Such an interval, especially when glycosuria is prevented by malaise and refusal of food, can decide nothing with the pancreas remnants mentioned, though permitting some observations after total pancreatectomy as in Carlson's experiments. Other attempts were made with successive operations, in the hope that each might be so easy as to avoid abortion, also by leaving a duodenal remnant and a subcutaneous graft early in pregnancy, with the idea that diabetes

might be brought on later in pregnancy by simple removal of the graft. All such attempts failed for one cause or another. It is sometimes possible to remove considerable portions of pancreas, even in the later stages of pregnancy, without accident, just as other abdominal operations are often feasible; but any pancreatic resection to the point of diabetes seems absolutely incompatible with continuance of gestation. The possibility that this disturbance may be due to the sudden deficit of the internal pancreatic function was excluded by two sets of controls; first, the results are just as bad when the entire uncinate process is left as a subcutaneous graft in addition to the usual pancreatic remnant; second, pregnant dogs ordinarily survive a week of fasting and phlorizination without abortion, though' the glycosuria and acidosis apparently represent a greater disturbance of carbohydrate metabolism than that following the pancreatic operations mentioned.

An attempt to test the influence of the increased food requirement and lactose formation of the lactation period was made as follows.

Dog C3–90, mongrel, in excellent condition, weighing 20.5 kilos, was found pregnant in an operation on May 25, 1916, when the splenic process and body of the pancreas down to the main duct were removed, with the idea that diabetes might later be produced by a very easy operation for removal of the uncinate process. The pregnancy continued uneventfully, but by mistake too long an interval was allowed, and on June 22 the dog gave birth to eight healthy puppies. She was an excellent mother and the puppies all thrived until, on June 29, the uncinate process was removed in an operation requiring only a few minutes. There was quick recovery from the brief etherization, and the dog showed the usual devotion and nursed the puppies immediately on being returned to the cage. She acted well and lively but ate very little on the following days, during which time the pups continued to nurse though the mother paid less attention to them. By July 4 her appetite was fully restored, but she refused to have anything further to do with the pups, and even injured them if they approached to nurse. Part of the trouble may have been due to tenderness about the abdominal wound, but there seemed also to be a genuine breaking up of the physiology and psychology of lactation by the operation. Milk rapidly disappeared from the breasts. Diabetes remained absent notwithstanding glucose feeding. Whether the disturbances observed were peculiar to the pancreatic operation or might follow any other abdominal interference, the experience showed that this method was not applicable for studying the influence of lactation upon diabetes.

Numerous microscopic examinations have been made of the pancreas of dogs in various stages of pregnancy and of a few during lactation, without the finding of any departure from the normal in any respect.

It was evident that a satisfactory study was possible only through the occurrence of pregnancy in animals already diabetic, so as to reproduce exactly the conditions encountered in diabetic women. Properly prepared animals with potential diabetes are normal in their entire behavior, and only the unfavorable laboratory conditions made the experiment difficult. It was hoped that cats might be particularly suitable for the purpose, but in the only instance in which a partially depancreatized cat became pregnant the experiment ended in failure, as follows.

Cat A1–84.—Female; strong adult; weight 3.5 kilos. December 20, 1913, pancreatic tissue was removed, not quite to the point of producing diabetes. The cat remained continuously free from glycosuria on meat, bread and milk diets thereafter, but had a permanently lowered tolerance, as shown by the production of glycosuria in subcutaneous glucose tests by doses between 1 and 1.5 grams per kilo. Impregnation occurred February 18, 1914. Thereafter the diet was meat and milk, mixed with as much lactose as the animal could be induced to take. Considerable carbohydrate could thus be given, though cats usually object strongly to the sweetness of glucose or saccharose. About the middle of March the animal was noticed to be weak and unwell. Abortion occurred March 16 and death March 18. There was no diabetes and the pancreas remnant was normal. The fatal outcome was almost certainly a sugar intoxication (4), with no specific relation to the partial pancreatectomy or to diabetes. Cats may possibly prove to be well suited to pregnancy experiments in diabetes, but they are as a rule an unfavorable species for carbohydrate over-feeding.

Far more numerous attempts were made with partially depancreatized dogs during three years, but all failed owing to the unfavorable environment. Finally a successful experiment became possible in dog B2-00, which was particularly valuable for the purpose because of the long previous records which had established the tolerance accurately. These observations have been reported in previous papers, especially no. 3 of series I (5) and no. 1 of series II (6).

A series of operations beginning in 1913 had made the dog nearly diabetic, but after the removal of an additional 0.8 gram of pancreatic tissue on September 6, 1916, it was still impossible to maintain glycosuria with the heaviest bread and

Dog B2-00.   .

Tests with Feeding 100 Grams Beef Lung, 200 Grams Bread and 150 Grams Glucose.

DATE	WEIGHT	PLASMA SUGAR	Hb.	URINE		REMARKS
				Volume	Glucose	
1916	*kgm.*	*per cent*	*per cent*	*cc.*	*per cent*	
November 21	13.4	0 106	101	18	0	Before feeding
		0.123	97	16	0	2 hours after feeding
		0.156	98	24	Faint	4 hours after feeding
		0 128	96	38	Faint	6 hours after feeding
November 23		0 092	95		0	Before feeding same diet as Nov. 21
		0 095	88	13	Faint	2 hours after feeding '
		0 139	92	No	urine	4 hours after feeding
		0.130	93	105	Faint	6 hours after feeding
December 16		Removed 0.1 gm. pancreatic tissue. Dog pregnant				
December 30	15 6	0 085			0	Before feeding
		0 345		212	3.45	2 hours after feeding
		0 455		192	6 46	4 hours after feeding
		0 500		68	5.89	6 hours after feeding
1917						
January 7	12.9	0 125		7	0	Before feeding. After abortion
		0 400		25	7.13	2 hours after feeding
		0.400		35	6 68	4 hours after feeding
		0 333		33	8.00	6 hours after feeding
February 23	16.3	0 116			0	Before feeding
		0 238		18	0.63	2 hours after feeding
		0.244		17	0.57	4 hours after feeding
		0.232		22	0.69	6 hours after feeding
May 17	15.6	0 130			0	Before feeding. (Early pregnancy)
		0.346		39	4.16	2 hours after feeding
		0.435		73	7.32	4 hours after feeding
		0.384		44	4.82	6 hours after feeding
July 11	14.9	0.106			0	Before feeding. (Late pregnancy)
		0 322		35	4.72	2 hours after feeding
		0.304		65	3.95	4 hours after feeding
		0.312		68	5 58	6 hours after feeding
July 19	13.0	0.077		0	0	Before feeding. (After delivery)
		0.200		23	0.72	2 hours after feeding
		0.218		16	0.92	4 hours after feeding
		0.232		13	0.95	6 hours after feeding

DATE	WEIGHT	PLASMA SUGAR	Hb.	URINE		REMARKS
				Volume	Glucose	
1917	*kgm.*	*per cent*	*per cent*	*cc.*	*per cent*	
August 9	12.9	0.081			0	Before feeding
		0.164		18	Very faint	2 hours after feeding
		0.109		36	Faint	4 hours after feeding
		0.135		17	Faint	6 hours after feeding
October 5	13.5	0.109			0	Before feeding
		0.159		17	0	2 hours after feeding
		0.145		36	Faint	4 hours after feeding
		0.152		46	0.31	6 hours after feeding
November 22	14 0	0 109			0	Before feeding
		0 204		24	Faint	2 hours after feeding
		0.238		36	0.39	4 hours after feeding
		0.125		24	0 44	6 hours after feeding

glucose feeding. Pregnancy then became evident, though the exact time of its beginning was unknown. Tests of the tolerance were performed by feeding, as described below. As diabetes remained absent with advancing pregnancy, there was danger that the entire result would be negative.

Accordingly, on December 16, 1916, 0.1 gram pancreatic tissue was removed, in an operation so short and easy that the dog did not even lose appetite for the day. Diabetes resulted, as proved by the fact that plain bread and soup feeding now maintained slight glycosuria (0.4 to 0.8 per cent, in 400 to 680 cc. urine). December 26 on this diet the plasma sugar was 0.091 per cent before feeding, 0.182 per cent four hours after. The special feeding test was repeated in late pregnancy on December 30.

As an additional experiment, it was attempted to learn whether the pregnant dog with latent diabetes had any special tendency to acidosis. Therefore nothing was fed on December 31, and only 300 grams suet daily on January 1, 2 and 3 (1917). Abortion then occurred, though the dog appeared only slightly unwell and remained free from acetone bodies in urine and blood, both before and afterward. The plasma sugar was constantly normal (0.089 to 0.105 per cent) after omission of carbohydrate, both before and after abortion. The carbohydrate tests had seemed harmless, and it may be possible that the fat feeding was responsible for the abortion even without acidosis.

January 27, the special feeding test was repeated as a control in the non-pregnant condition. The existence of diabetes was unmistakable. The diet was then changed to meat to prevent downward progress, and the dog was left most of the time with a male in the hope of a second pregnancy. The plasma sugar meanwhile remained normal (0.087 to 0.113 per cent) on the carbohydrate-free diet. The special feeding test was repeated on February 23 and May 17.

Pregnancy then became manifest, and the feeding test was repeated in this condition on July 11. June 28 and July 2, the plasma sugar on the meat diet was normal (between 0.075 per cent before feeding and 0.105 per cent after feeding).

July 12, less than 0.1 gram of pancreatic tissue was removed without appreciable disturbance.

Three pups were born on July 16. They were probably 2 or 3 days premature, and were all dead by July 19 owing to failure to nurse properly. This result may have been independent of the experimental conditions, for the dog was of a type which often has troubles with puppies.

The special feeding test was repeated on August 9, October 5 and November 22.

Owing to the perfect manner in which the dog bore the experiments through two pregnancies, the questions at issue received very satisfactory answers as follows:

a. In the first instance, pregnancy failed to produce diabetes in an animal which was so close to the verge that diabetes resulted from the removal of 0.1 gram additional pancreatic tissue. Against an assumption that the diabetes here was due largely to the pregnancy and only partially to the pancreas operation is the fact that diabetes persisted after termination of the pregnancy. Also in the second instance, when the dog was known to have mild diabetes but was free from symptoms on meat diet, pregnancy failed to produce either glycosuria or hyperglycemia on this diet.

b. The removal of a bit of pancreatic tissue in the latter part of each pregnancy supplemented the observations of the negative effects of gestation and especially demonstrated the absence of vacuolation of the islands first when the animal was not quite diabetic and second when she was mildly diabetic. The absence of such hydropic changes with latent diabetes corresponds fully to the experience in non-pregnant animals.

c. The passage of any appreciable quantity of pancreatic internal secretion from the fetuses to the mother is disproved by two facts. First, the occurrence of diabetes following the operation of December 16, 1916, was not prevented; in other words, there was no transfer of pancreatic hormone sufficient to compensate for the loss of 0.1 gram of maternal pancreatic tissue. Second, the feeding tests demonstrated an actual aggravation of the diabetes during pregnancy. Test meals were used instead of intravenous glucose injections because according

to previous experience (7) they afford a more accurate index of the diabetic condition and also because they seemed to promise less danger of abortion or other accidents. Attention may be called to the results of these tests as shown in the table.

The test meal consisted of 100 grams beef lung, 200 grams bread and 150 grams glucose. Only slight hyperglycemia and faint glycosuria resulted from this diet on November 21 and 23, when the dog was nondiabetic. The removal of 0.1 gram pancreatic tissue on December 16 produced a radical change, so that the hyperglycemia and glycosuria in the test of December 30 were of plainly diabetic character. The tolerance was fully as low on January 7, after termination of pregnancy, but the dog was still unwell from the recent abortion. The test of February 23 showed a considerably better tolerance as judged by both blood and urine.

May 17, early in the second pregnancy, the test showed a well-marked fall in the assimilation, and the result was not greatly different in the late stage of pregnancy on July 11. The bit of pancreas removed on July 12 was so tiny that it apparently had little effect upon the tolerance. At any rate, in spite of this operation, the assimilation on July 19, three days after normal delivery, showed a decided improvement. This improvement was still greater on August 9, after all puerperal disturbance had subsided. It was also maintained up to October 5, the length of the experiment and the uniformity of results being thus sufficient to exclude accidental fluctuations of tolerance.

CONCLUSIONS.

1. No positive influence of the sex glands upon diabetes was demonstrable by extirpation experiments. Also no anatomic changes in the pancreas were perceptible with pregnancy or lactation.

2. Observations upon a partially depancreatized dog during pregnancy are opposed to the view that any appreciable quantity of internal pancreatic secretion passes from the fetus to the mother.

3. A distinct lowering of carbohydrate assimilation was shown during pregnancy. This was not clearly associated with the increase of metabolism, in the sense either of increased food requirement or new tissue formation, for it seemed approximately the same in early

and late pregnancy and was also evident during the illness following abortion. It may therefore be regarded chiefly as a toxic manifestation and thus classifiable with the influence of infection. The effect is relatively slight, because pregnancy failed to produce diabetes in a dog where the removal of 0.1 gram pancreatic tissue sufficed for the purpose, and also after the dog was demonstrably diabetic on carbohydrate diet pregnancy gave rise to neither glycosuria nor hyperglycemia on carbohydrate-free diet.

The tests with partial pancreatectomy, which affords the most exact method of study, suggest that Carlson's results in totally depancreatized dogs are to be interpreted as cachexia. Clearer information of the influence of pregnancy upon the internal pancreatic function is also afforded by the freedom from the variables which enter into clinical cases. The slight tendency to aggravation of the diabetes and the ready control by diet support Joslin's experience of the feasibility of completion of pregnancy by diabetic women under suitable conditions of treatment. If the toxic factor is the principal one, as suggested, the possibility remains that the injurious action in some women may be considerably greater than in dogs and accordingly may require more radical measures.

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PRESERVATION OF STOCK CULTURES OF BACTERIA BY FREEZING AND DRYING.*

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The object of this communication is to present the results of the prolonged preservation of stock cultures of bacteria after freezing and drying, and to describe the method used.

The benefit of a method that will assure the preservation of cultures of bacteria without frequent manipulation is self-evident. As type organisms are isolated or identified it is desirable to keep the original state of virulence or to retain their cultural characteristics. In addition to the danger of contamination from frequent manipulation, there is the well known tendency for many bacteria to lose their virulence or other biological characteristics from repeated transfers on artificial media. During the course of many investigations it is often of advantage to keep all the bacteria isolated until there is time for more detailed study. A method that will permit the shipping of cultures from one city or country to another without the bulk of culture media, or without danger of breakage or death of the bacteria, has obvious advantages.

The principle of keeping bacteria in a dormant state by desiccation after freezing has been known for several years. Shackell (1) was one of the first to call attention to its advantages. He showed, first, that after freezing, substances such as tissue could be uniformly dried; second, that serum retained its complement and antibody activity unaltered for weeks; and third, that rabies virus in rabbit brains did not lose its virulence when kept in this manner. Hammer (2) dried bacteria previously frozen on strips of filter paper, and kept them from 54 to 57 days. Controls, dried without freezing, were killed immediately. Shattock and Dudgeon (3) showed that organisms dried on charcoal without freezing were usually killed in from 4 to 40 days. *B. pyocyaneus*, however, lived for at least 7

* Read before the American and Canadian Section of the International Association of Medical Museums, New York, N. Y., April 1, 1920.

months. Macfadyen (4) and Macfadyen and Rowland (5) demonstrated that practically all bacteria as well as yeast could be frozen and reduced to a temperature of liquid hydrogen, *i.e* −252°C., and later recovered in a viable and unchanged condition. Rogers (6) finally applied the principle of freezing and drying on a large scale so that mass cultures of lactic acid-forming bacilli could be preserved for commercial purposes. He suggested the use of this method for the preservation of stock cultures for the laboratory.

For the past 6 years we have been using this method in the preservation of stock cultures of streptococci and pneumococci. Recently upon attempting to recover these bacteria it was found that if they were originally prepared under proper conditions they were viable after a period of from 2 to 4 years. Comparison of the tubes from which the organisms could not be recovered, with those from which they were easily recovered, showed that the physical state of the dried material had much to do with the viability of the organisms. For example, of twenty-two tubes in which it was possible to recover bacteria, nineteen were in a condition of "dry foam" and three in a condition of "flaky gum." Among eleven with which failure occurred, nine were distinctly gummy, one showed a "flaky gum," and one a "dried foam." This physical state with the accompanying success or failure was present immediately after the organisms were dried or after 4 years. The results showed that it was necessary to maintain the frozen state until drying was complete; this led to the devising of the present method in which the tubes are immersed in glycerol which acts as a medium for the conduction of cold from a salt-ice mixture.

Method.

Apparatus (Text-Fig. 1).—A desiccator (*D*) is chosen with broad opposing surfaces on cover and stop-cock. It is usually necessary to regrind all opposing glass surfaces. On the inner side of the desiccator is fastened with adhesive plaster a small mercury manometer (*M*) with one open and one closed end. In the upper part of the desiccator is a receptacle (*R*) for the desiccating salt; if phosphorus pentoxide (*Ph*) is used this container can be of copper. In the bottom of the desiccator is placed about 4 cm. of commercial glycerol (*G*). A pan (*P*) is provided, large enough to hold the desiccator and

a salt-ice mixture (S) which is packed around the latter. It is necessary to have a mechanical pump that will give a vacuum as low as 2 or 3 mm. of mercury; preliminary exhaustion of the larger part of the air can be conveniently carried out with the ordinary water pump.

Preparation of Cultures.—The object to be attained is a maximum number of young actively growing forms of the bacteria in a minimum
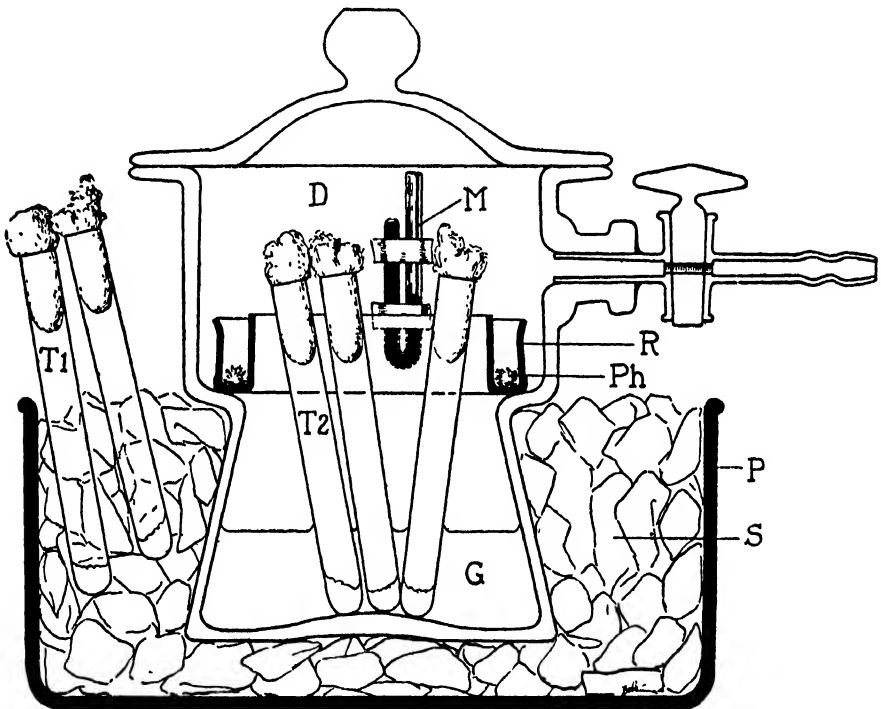
TEXT-FIG. 1. Diagrammatic cross-section of the apparatus used for freezing and drying bacterial cultures for preservation. *D*, desiccator; *M*, mercury manometer; *R*, copper receptacle for the phosphorus pentoxide (*Ph*); *G*, glycerol in the bottom of the desiccator; *P*, pan for the salt-ice mixture (*S*); *T 1*, position of a tube of culture during freezing; *T 2*, position of a tube of culture during desiccation.

of fluid. It is necessary to have enough fluid to form a small amount of ice. If fluid media are used it is well to grow the organisms for 15 to 18 hours, centrifugalize the culture, and discard most of the supernatant broth. The concentrated bacteria can then be pipetted into a number of small tubes and frozen. With certain bacteria it

is necessary to use the growth from solid media.   In this case the growth should be scraped off into a small amount of condensation water or of broth that has been added, and the suspension subsequently pipetted into tubes.   We have found it convenient to use small tubes, about 10 cm. long and from 5 to 10 mm. in diameter.

Manipulation.—After the culture has been placed in the small tubes as above described the desiccator containing the glycerol is set in the pan containing the salt and ice mixture; the tubes as well are immersed in this mixture and the cultures frozen (position *T 1*, Text-fig. 1).   Simultaneously the temperature of the glycerol is reduced to minus 4–6°C.   After careful lubrication of the desiccator cover and stop-cock and after the glycerol has reached a proper temperature and the bacterial suspension is well frozen, the tubes are immersed in the glycerol (position *T 2*), the phosphorus pentoxide is put in the copper pan, and the cover placed securely on the desiccator.   The air is then exhausted from the desiccator, first with an ordinary water pump and finally with the high vacuum pump, during all this time the desiccator being kept in the salt-ice mixture.   When the proper degree of vacuum has been obtained the whole apparatus is placed in the ice box where it is left until desiccation is complete.[1]   This .time, in our experience, is usually about 12 hours.   When it is certain that desiccation is complete the tubes are removed from the desiccator, the cotton stoppers pushed down into them, and melted paraffin is poured in until the tubes are thoroughly sealed.   It is usually necessary to paraffin the tubes two or three times in order to insure complete sealing.   If it is desired to keep the organisms for many years the tubes may be sealed by melting and fusing the open ends of the tubes.   After the tubes have been well labelled, they can be kept at room temperature, preferably in the dark.

The appearance of the properly dried culture is that of a very light, spongy, flaky material.   If it is separated from the sides of the tube it can be shaken about and looks like dried lather made from shaving soap.

Recovery of Organisms.—The organisms may be recovered in one of several ways.   For such bacteria as the cocci, and those of the

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[1] If a frigo ice box is available it is advisable to place the desiccator at a temperature below zero.

colon, typhoid, and dysentery group, as well as other organisms that grow easily in broth, after the removal of the stoppers, broth may be added directly to the dried powder and the tubes incubated. Organisms like meningococci or others that grow better on solid media are best recovered by picking up a small amount of the dried material on a platinum loop and smearing it over the surface of suitable media, after it has been moistened in the water of condensation.

Results obtained by this method with various types of organisms are given below.

Streptococci and Pneumococci.—Out of fifteen strains of non-hemolytic streptococci kept from 22 to 40 months, all except two showed growth like that of the original culture, with similar fermentation reactions. These two, however, showed similar types of fermentation reactions with all the subcultures recovered; it is therefore probable that the change in fermentation occurred before the organisms were frozen. In the strains which originally showed a moderate degree of virulence, the virulence was maintained after the recovery of the organism. Four strains of hemolytic streptococci have been recovered after a period of from 42 to 51 months. Three strains of pneumococci were preserved in this manner and upon recovery several months later had retained their original virulence and reaction to type serum. One of the strains that had been accustomed to grow on 75 per cent bile retained this property in the subcultures.

Meningococci.—It has been found best to grow meningococci on solid media, scrape off the growth in a small amount of water of condensation, and freeze the resulting suspension. An experiment was performed to determine how small an amount of meningococci might be preserved in this manner; it was found that one loopful suspended in 0.2 cc. of broth or condensation water could be kept for a period of at least 2 months following desiccation. No tests were made after longer intervals. In the recovery of meningococci it has been found much more satisfactory to smear the dried powder upon the surface of freshly prepared blood agar or dextrose serum agar; the addition of blood dextrose broth to the dried powder has yielded more uncertain results. *Bacillus influenzæ* is best treated in the same manner as meningococci.

Other Organisms.—Typhoid, paratyphoid, and dysentery bacilli have been frozen following growth either in broth or on solid media. They were easily recovered by adding broth to the dried culture.

Other organisms of the bacteria group have not been tested because it is felt that those mentioned above represent the various types. There are few bacteria more delicate and more difficult to maintain in stock cultures than the meningococci and *Bacillus influenzæ.* Attempts have been made to preserve the spirochete of relapsing fever recovered from the blood of rats inoculated with these organisms, but the freezing seems to be sufficient to kill completely all the organisms. It is probable that spirochetes in general are not susceptible to preservation in this form, as a number of different strains have been shown to succumb to freezing. No work has been carried out by us with filterable viruses, but Harris and Shackell (7) have shown that the virulence of rabies virus is retained in this manner. Rous[2] has been able to preserve the virus of chicken sarcoma by a similar method for 7 years.

SUMMARY.

Attention is called to the fact that bacteria may be preserved for a long time by desiccation in the frozen state. It has been shown that it is necessary to maintain the frozen condition until desiccation is complete; if the fluid melts before the moisture is completely removed, the organisms are killed, probably because of the concentration of the salts upon the surface of the bacteria. By the simple expedient of immersing the tubes of organisms in glycerol contained in a desiccator and subsequently keeping the whole apparatus in a salt-ice mixture until drying is complete, the organisms are easily maintained in the frozen state, and dry properly. Bacteria preserved in this manner retain their cultural, biochemical, and immunological characters for prolonged periods.

[2] Rous, P., personal communication.

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6. Rogers, L. A., *J. Infect. Dis.*, 1914, xiv, 100.
7. Harris, D. L., and Shackell, L. F., *J. Am. Pub. Health Assn.*, 1911, i, 52.

THE FOOD REQUIREMENTS OF CHILDREN.

I. TOTAL CALORIC REQUIREMENTS.*

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In the course of our metabolism observations on young children many interesting facts have been brought out with respect to the general food requirements as to total calories, distribution of the calories as fat, carbohydrate and protein, and other factors in the diet which must be considered. One notes in the literature on the food requirements of growing children a great difference of opinion among the various observers who have studied the subject. Many writers have taken up this problem from only a single point of view such as the total caloric requirement, of the basal requirement, or the food needs in respect to one or another of the accessory food substances, the so-called "vitamins."

We have, therefore, thought it desirable to consider in its different aspects the whole subject of the food requirements of the child during the entire period of growth.

We shall discuss in this paper the total caloric requirements of children and the different factors which must be reckoned with in estimating the total calories. In succeeding papers we shall take up the proper distribution of the calories of the food as fat, carbohydrate and protein, also other important factors in the diet such as roughage, the difference in growth-promoting properties of the different proteins, the supply of essential inorganic salts and the accessory food substances or vitamins.

At various times and by various authors each of the different factors mentioned has been discussed, but as yet no one has attempted to correlate them all and study the food needs of the child in the entire

* Read at the annual meeting of the American Pediatric Society, May 30 and 31, June 1, 1920.

aspect.   The estimates of total caloric requirements of children made by some authors have been purely hypothetical, while others have been based on the requirements of adults.

Observations made during the last few years, especially in this country, have thrown new light on the question of the total calories needed by the growing child.   It is essential to review not only this published work, but also most of the older observations, made chiefly in Germany.   From these older observations conclusions have been drawn which do not seem to be warranted either by the number of children studied or the conditions under which they lived.   For instance, one author made but a single observation on one child; another's observations were made almost entirely on his own children; those of a third were made on a considerable group of young children who were inmates of an orphan asylum, most of whom were very much under weight; a fourth studied three girls, two of whom were very much over weight.

It is recognized by all who have studied this subject that even in normal healthy children there are very considerable individual variations in food requirements.   These variations are certainly much greater in abnormal children or in those studied under unusual conditions.   Yet the small number of observations made by some of the German authors referred to are quoted over and over again in textbooks and periodical literature and have been made the basis of very broad and widely accepted deductions.   In the light of more recent studies made on a larger scale, some of these deductions seem to be erroneous and misleading.

Component Factors of Caloric Requirements.

In calculating the total caloric requirement of a child there must be taken into account (1) the basal requirement, (2) growth needs, (3) needs for muscular activity, and (4) the food values lost in the excreta. The first of these, the basal requirement, is fairly uniform for children of the same weight.   Growth requirements diminish rapidly from birth to the end of the third year and vary but little from this time until the tenth or eleventh year, after which they undergo a marked increase, which is maintained until growth is practically complete. The variation of this growth rate is fairly uniform with all children.

Muscular activity, however, varies enormously with different children, but in general tends to increase steadily from birth to puberty. The caloric value of the foodstuff lost in the excreta is subject to very little variation in healthy children of the same age, unless there are marked differences in the diets.

Basal Requirements.— A great deal of accurate information has been accumulated regarding basal requirements, that is, the needs of the body at complete rest. This knowledge has been obtained by calorimetric observations made by Benedict and Talbot, DuBois, Murlin and others in this country and by Voit and Pettenkoffer, Rubner and Heubner, Schlossman and Murschhauser, and Magnus-Levy and others abroad. Some have based their comparison of individuals on the body surface, but the body weight has been most frequently employed. Many studies have been made of the basal metabolism of adults, both in health and disease, and a large number of observations have been made on infants, especially the new-born. The intervening period, from the age of one year until the end of puberty, has not been so generally investigated. Talbot and Benedict are the only observers who have studied this entire period systematically.

Benedict[1] has recently presented the results of about 250 observations made by himself and Talbot on children of both sexes, more than half of whom were over one year of age. The range of individual variation in the values obtained was considerable, but the number of observations made is so large that the data seems sufficient to warrant us in accepting their curves as representing the average for the period of growth.[2] They have shown that the basal metabolism per kilo of body weight is at birth "specifically low," that it rises rapidly until the body weight has reached seven to nine kilos and then diminishes slowly to adult life (Charts 1 and 2). There is some difference between the basal needs of boys and girls. After a weight of ten kilos (about one year) is reached the basal requirement of boys exceeds that

1. Benedict, F. G.: Boston M. & S. J. **181**:107 (July 31) 1919.

2. In a personal communication Talbot stated that the curve for the latter years of childhood may need some revision, since up to the present time the number of observations has not been sufficient to establish definitely this part of the curve.

of girls until a weight of about thirty-five kilos is reached (at about 11 years of age), when the basal needs of girls for a time exceed those of boys. Benedict and Talbot found that the basal caloric requirement increases with age but the relationship is best expressed as calories per unit of body weight or of body surface. There was, in their opinion, no closer consistency in the caloric values when expressed per
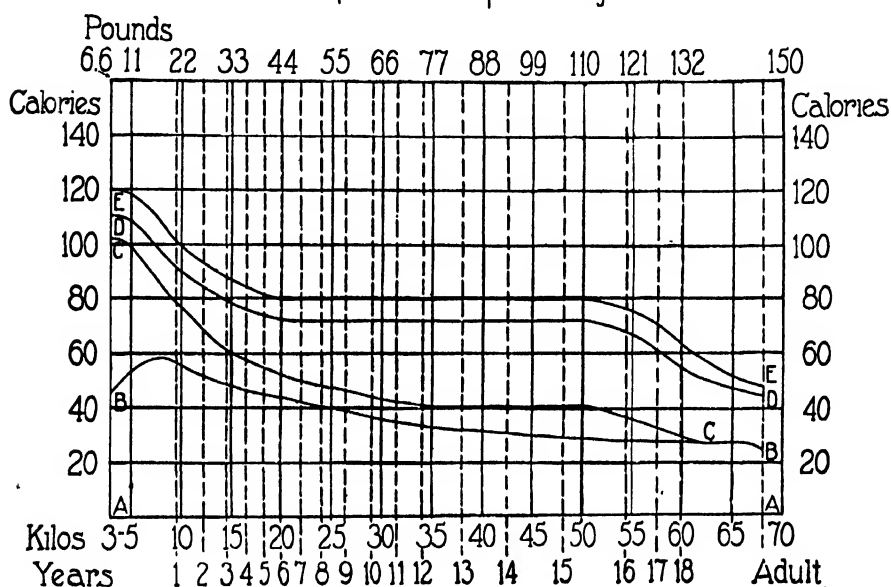
Fig. 1.—The solid vertical lines indicate weights in kilos; the broken lines, approximate weights at each year of age. The space between lines AA and BB shows allowance for basal metabolism; between BB and CC, that for growth; between CC and DD, that for muscular activity; between DD and EE, food values lost in excreta. The space between the lines AA and EE shows the total caloric allowance per kilo.

unit of body surface than when expressed per unit of body weight. In our discussion, therefore, we shall use the more convenient standard—the unit of the body weight.

Magnus-Levy[3] in 1899 studied the basal metabolism of twenty-five children of 2 years of age and upward, but the ages studied were widely scattered and the values obtained for different individuals

3. Magnus-Levy and Falk: Arch. f. Anat. u. Physiol., 1899, p. 314.

show wide variation. For the most part his values are higher than those obtained by Benedict and Talbot.

DuBois[4] made studies of the basal metabolism of eight boys between 12 and 14 years of age. The values he obtained are also somewhat higher than those of Benedict and Talbot, but are lower than those of Magnus-Levy. Two years later he[5] studied the same boys and found that the basal metabolism per kilo had diminished but that the values obtained fell on the curve which he had deduced, using
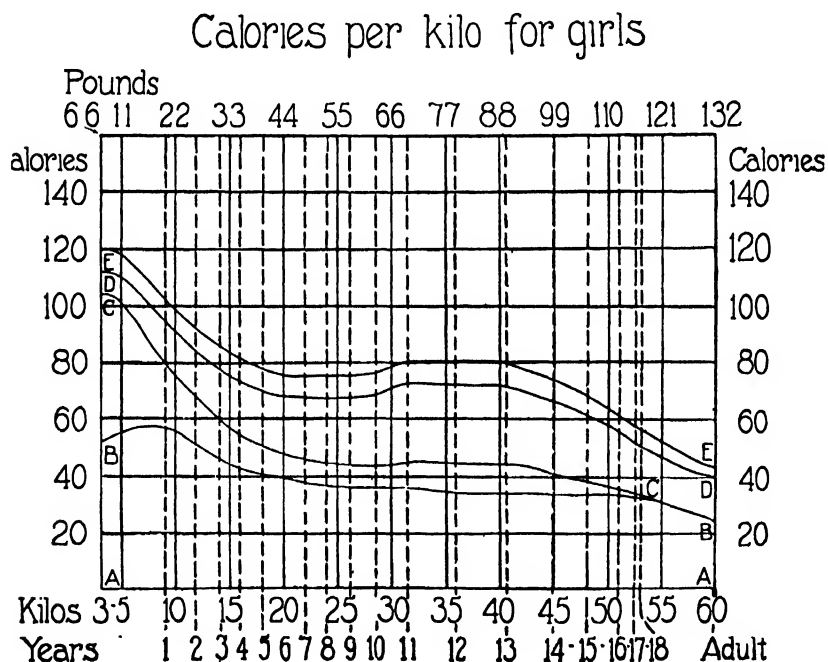
FIG. 2.—The vertical and curved lines have the same significance as in Chart 1.

his own observations and those of others. The reduction in basal calories per kilo in case of these boys was only the normal reduction which takes place steadily with increasing years.

On the whole, it seems reasonable to accept the values for basal requirement given by Benedict and Talbot as representing what they

4. Dubois, E. F.: Arch. Int. Med. 17:887 (June) 1916.

5. Olmstead, W. H., Barr, D. P., and DuBois, E. F.: Arch. Int. Med. 21:5 (Jan.) 1918.

have termed "the irreducible minimum" which must be supplied to the human organism for maintenance.

Growth Requirements.—We shall not at this point consider the special growth needs, such as necessary vitamins or the need of supplying proteins which have the essential amino-acids, but only the energy requirements for growth which are met by the fat, carbohydrate and protein furnished in the ordinary articles of food. The caloric requirements for growth must, it is obvious, be greatest when growth is most rapid and diminish at the period when growth is slower. The rate of growth is most rapid during the first year of life, diminishing greatly in the second year. It is again accelerated at the approach of puberty. The diminution in the growth rate during the early years and the acceleration during the later years are indicated by the annual increase in weight and height from birth up to the time when the body is completely grown. This average increase is shown for both sexes in Chart 3. If we combine the curve representing the annual increase in weight and that for the annual increase in height we obtain a curve which may be taken to represent the annual increase in the size of the body during the period of growth. This rate of increase steadily increases in both sexes after the age of 10. In boys by the sixteenth year and in girls by the thirteenth year it is nearly twice as great as it is at ten years.

In calculating the total caloric requirements in the past it appears that sufficient consideration has not been given to these variations in the rate of growth. The increase in the body's needs for growth is not uniform as age advances from early childhood through the period of adolescence.

Using as a basis for calculation the annual increase in weight, it is possible to estimate very approximately the number of calories needed for growth by a child at any given age and weight. Rubner[6] estimates that about 80 calories per diem are needed to increase the weight of the human body one kilo in one year. This value multiplied by the average increase in kilos per annum would give approximately the number of calories needed daily for growth. On this basis the daily caloric need for normal growth for boys is over 200 calories during

6. Rubner, M.: Das Problem der Lebensdauer und seiner Beziehung zu Wachstum und Ernährung, 1908.
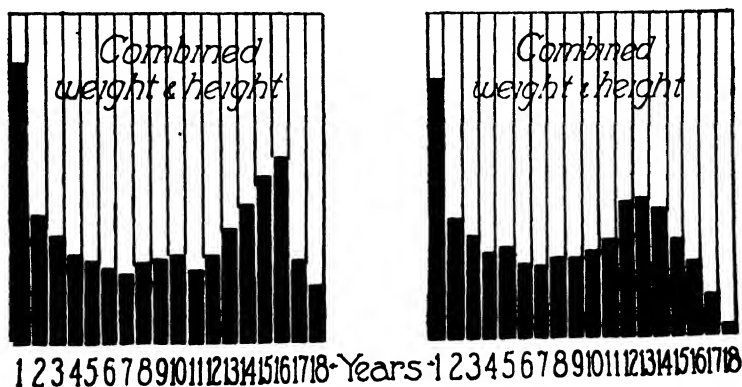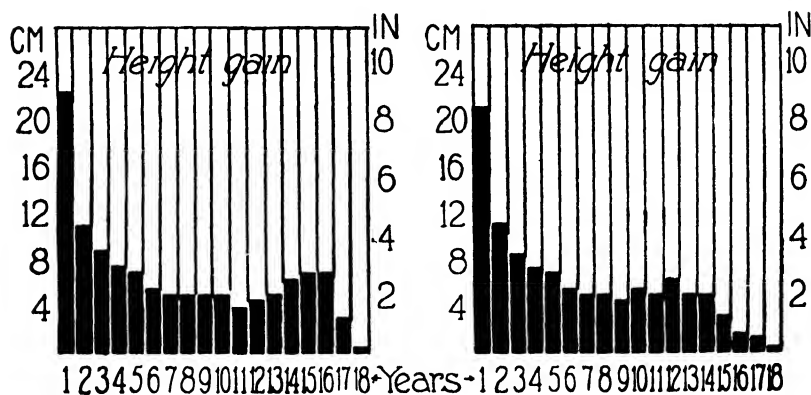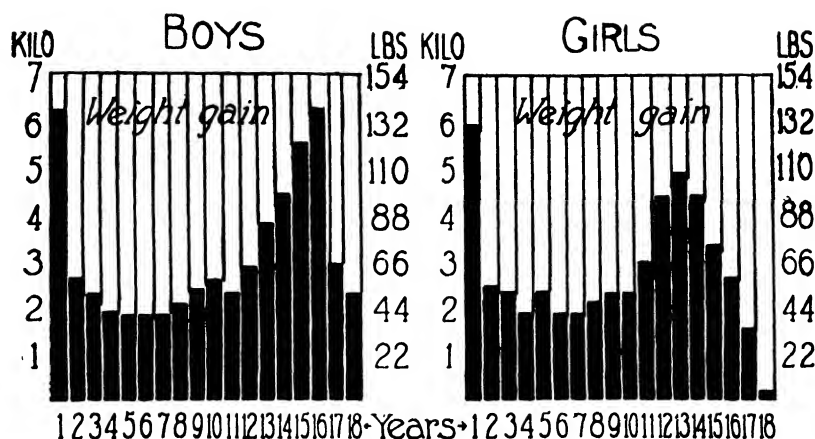
FIG. 3.—The heavy black verticals of the two upper charts show the annual gain in weight in kilos and pounds, and the gain in height in centimeters and inches, for both sexes. The lowest charts show values obtained by combining the two.

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the second year; between 4 and 8 years it diminishes to about 150 calories, then rises steadily, reaching over 500 calories daily during the sixteenth year, after which it rapidly falls (Chart 5).

Requirements for Muscular Activity.—It is difficult to estimate with even approximate accuracy exactly how many calories should be allowed for muscular activity. Allowance must be made for all activity, whether productive or unproductive. Even the energy expended in the process of digestion must be taken into account in estimating these needs. Benedict states that the energy needed to digest the food taken uses up about 6 per cent. of the total calories of the diet. This he terms the "cost of digestion." It is a factor not usually considered but is important. Like the food value lost in the excreta it is one of the inevitable losses and one for which allowance must be made in estimating the total caloric requirement.

The requirements of individual children for activity differ very greatly, more widely than they do in any other respect. A nervous, lively, energetic child will certainly use up more calories in activity than one of quiet, placid temperament and indolent muscular habits. How much the difference is between the actual needs of these two types we can, with our present knowledge, only conjecture.

Lusk[7] has estimated that during the period from four to fifteen years the very active child requires a little more than double the total calories of a quiet child. Since three of the four factors which make up the total calories, namely, basal requirement, growth requirement and the food value lost in the excreta, are nearly the same for these two types of children, it would appear that practically all of the increased allowance which Lusk proposes, or considerably more than half the total food taken, is used up by the very active child in muscular activity. For young children this seems excessive. It certainly cannot be taken as an average. At any age it is probably applicable only to a small group.

Lusk allows for the child of moderate activity about one-third more calories than for the quiet child. This value is probably more nearly correct for the younger children, but the allowance is not enough for boys after 12 years of age and probably not for girls.

7. Lusk, G.: J. A. M. A. **70**:821 (March 23) 1918.

A steady increase in the calories which are needed for activity must be allowed as age advances. As soon as a child learns to run about a great increase in activity takes place, and with the normal healthy child activity steadily increases with each year of life, at least up to the period of adolescence.

DuBois estimates that walking at a moderate pace on the level uses three times the basal requirement. On this basis, a boy of about 30 kilos weight, that is, of about 10 years of age, walking on the level for two hours, would require an increase of 270 calories to supply energy for this activity.

The great difference in food requirements of children because of the difference in their activity is not in most cases sufficiently taken into account. If the increased caloric need of the active child is not supplied by the diet, growth inevitably suffers and, as Talbot[8] has pointed out with respect to infants, there is no gain in weight. The same consequence—failure to gain weight—follows when for any reason the digestive organs are not equal to the extra task imposed by increased ingestion of food. All these factors, and not simply the average needs as worked out for large groups, must therefore be taken into account when one is considering the needs of a single child or a small group.

Food Values Lost in the Excreta.—No accurate observations on this point have been published regarding children over 6 years of age. Our own studies lead us to the opinion that this factor varies less with age than do the other three factors which make up the total food requirement, and that in health a loss of calories equal to about 10 per cent. of the food intake is approximately correct for children of all ages. Some variation in children of the same age and weight will of course be seen, depending upon the amount and nature of the food, the care of preparation, on thoroughness of mastication, etc.

Extensive work done by the Department of Agriculture on the diet of adults has established quite definitely that the loss in the excreta, when a mixed diet is taken, averages about 9 per cent. of the total calories ingested. Benedict states that when a mixed diet is properly balanced and the digestion is normal, the loss in the excreta does not

8. Talbot, F. B.: Am. J. Dis. Child. 18:4 (July) 1919.

average more than 6 per cent.   Atwater and Sherman, who followed the metabolism of three six-day bicycle riders, found an average loss in the excreta of nine per cent. of the total calories taken.

Müller[9] made a careful study of the diets of thirty-two children from 2 to 6 years of age.   In order to determine the exact food value lost in the excreta, he dried the feces and urine of each child, as well as a sample of the composite food of each, and determined the exact caloric value of these substances by burning samples in a bomb calorimiter.   The average caloric value of the dried feces was 5.0 calories for each gram of dried matter, the range being from 4.6 to 5.4 calories per gram.   The urine showed a caloric value ranging from 8.7 to 13.9 calories for every gram of nitrogen which it contained, the average being 10.4 calories per gram of nitrogen.   The average daily caloric loss in the feces of the children observed by Müller was 5.9 calories per kilo of body weight.   The average loss in the urine was 4.6 calories per kilo of body weight.   This gave an average total daily loss in the excreta of 10.5 calories per kilo.   The average intake being 103.7 calories per kilo, the average loss in the excreta was, therefore, 10.1 per cent. of the intake.   The individual loss ranged from 8 to 14 per cent. of the total caloric intake.

In our own observations we have found that healthy children under 6 years of age taking a mixed diet have from 10 to 20 gm. of dried matter in the daily stools.   This represents a loss in the stools of from 50 to 100 calories, using as a basis for calculation Müller's average figure for the caloric value of dried feces.   According to our observations, which confirm those of other authors, children from 1 to 6 years of age taking a mixed diet excrete from 4 to 6 gm. of nitrogen in the urine daily.   Using Müller's average figure for the caloric value of urine, this indicates a loss of from 40 to 65 calories daily.   The combined loss in urine and feces by children from 1 to 6 years of age is, therefore, from 90 to 165 calories daily.   This is approximately 10 per cent. of the calories usually given at these ages, and, therefore, corresponds closely with the value suggested by Talbot for infants.

Using Müller's average caloric values for feces and urine, we have calculated the exact caloric loss through the excreta in five observa-

9. Müller, E.: Biochem. Ztschr. 5:143, 1907.

tions on normal children. The loss was found to range in these cases from 8.8 to 10.6 per cent. of the total calories of the food taken and the average was 9.6 per cent. We found that children suffering from a mild form of chronic intestinal indigestion had a loss through the excreta ranging from 10 to 15 per cent. of the total calories taken, while in cases of severe chronic intestinal indigestion the loss was at times as high as 25 per cent. of the total calories of the food taken. A summary of these findings is shown in Table 1.

In cases of severe chronic intestinal indigestion the total food value lost in the stools must frequently be much greater than the 25 per cent. estimated. For in such cases there is usually a very large loss

TABLE 1.

Average Nutritive Values Lost in Excreta.

Condition of Children	Number of Observations	Age in Years	Dried Weight of Feces Gm Daily	Calories Lost in Feces	Nitrogen in Urine, Gm Daily	Calories Lost in Urine	Total Calories Lost in Excreta	Calories in Food taken	Per Cent of Calories in Excreta	
									Average	Range
Normal..........	5	2–3 5	11 6	58	5 3	55	113	1,177	9 6	8 8–10 5
With mild chronic indigestion.....	10	1–3	14 5	73	4 9	51	124	1,008	12 3	10 0–14.5
With severe chronic indigestion.....	12	2 5–9*	21 1	106	5 8	60	166	865	19 2	12 9–24.7

* Some of the children in this group, although older, were much undersize and underweight and were therefore comparable to those in the other groups.

of carbohydrate through fermentation in the intestine. The chief products of this fermentation—gases and volatile fatty acids—do not appear in the dried matter of the stool and we have no convenient method of estimating them. Moreover, there is often some loss of protein due to putrefaction, which is represented in the stool mainly by volatile substances. How many calories these additional losses represent it is difficult to estimate.

On the whole, our estimate of 10 per cent. of the calories for loss in the excreta seems a reasonable average allowance for normal children taking a mixed diet. This allowance would certainly be high for nursing infants, however, since our observations have shown that

these infants utilize from 95 to 99 per cent. of the fat intake and a very much higher proportion of the protein intake than do artificially fed infants.

Total Calories.

A number of authors have made observations on individual children or groups of children to determine the total calories taken at different ages. A comprehensive survey of the literature on this subject up to 1917 has been made by Lucy H. Gillett.[10] In her report is given an extensive bibliography and a summary of such values reported by various authors as have been based on actual observations of food taken. Such observations are surprisingly few. Most of them have been made by the German authors and have been so widely quoted and have formed the basis of so many calculations for total calories per kilo that it seems worth while to go into detail regarding the children studied. There are objections to using the results obtained with almost all the small groups observed for the determination of the requirements of average healthy children living under normal conditions.

Hasse[11] calculated the calories taken by six girls belonging to well-to-do families. The ages of these children ranged from 2 to 11 years. All were in good health, but, as compared with our standards two were under weight, while the others were somewhat over weight. The two under-weight children, who were 2 and 5 years of age, took respectively 102 and 90 calories per kilo, rather high values for their ages. The children of 3 and $3\frac{1}{2}$ years took 77 and 81 calories per kilo, about the usual for their ages. The two older girls, 9 and 11 years of age, took only 65 and 61 calories per kilo, respectively, but these girls were considerably over average weight.

Herbst[12] also observed six children in well-to-do families. Three were boys, aged 2, 4 and 10 years. The youngest was considerably over weight, the other two practically the average weight for their ages. They took respectively 90, 87 and 66 calories per kilo. The

10. Gillett, L. H.: Publication 115, N. Y. Assn. for Imp. of the Cond. Poor, 1917.

11. Hasse, S.: Ztschr. f. Biol. **18:**553, 1882.

12. Herbst, O.: Jahrb. f. Kinderh. **46:**322, 1898.

other three children were girls in their eleventh, thirteenth and fifteenth years. The first two were very much over weight, the third somewhat over weight. They took respectively 45, 41 and 38 calories per kilo. These values are very low indeed, but the children observed were in a rather exceptional physical condition and data derived from the study of their food can hardly be used as a basis for estimating the needs of the average child. These observations, however, confirm the generally accepted opinion that the food requirements per kilo of overweight children are much lower than those of children of average weight for their age.

Müller[9] studied twenty-three boys and nine girls, each for a six-day period, and collected and analyzed the urine and feces of each child, as well as recording the exact amount and kind of food taken. The ages of the children ranged from 2 to 6 years. The children were reported to be healthy and free from nutritional disturbances, but all were inmates of an orphan asylum and appear to have been fair examples of what is commonly spoken of as "institution children." Nearly all of the children were under weight, the boys almost without exception markedly so. As might have been expected with children of this class, the intake per kilo was high, the average for the group being 104 calories per kilo. Two-thirds of the boys weighed between 11 and 15 kilos and these boys, all under weight, took on the average 113 calories per kilo. One boy of 4 years with the weight (12.6 kilos) of an average child of about 2 years, took 132 calories per kilo; another child, 6 years old, of about the same weight as the one just mentioned, took 126 calories per kilo.

The girls, who were not so much under weight as the boys, took on the average about 15 calories per kilo less than did the boys. These values correspond fairly well with the usually accepted ones for their ages, except in the single instance of one girl who was about the average weight for her age and who took only 71 calories per kilo.

Müller's observations were made in winter. He thinks that season should be taken into account in estimating the food needs. His results show that children who are much below normal weight require much more food per kilo than do normal children. All the facts concerning the children observed must be known in order to appreciate and properly interpret the data obtained. We are no more justified in

basing our estimates for normal requirements upon the results obtained with these underweight children than on the results with Herbst's over-weight girls.

Uffelmann[13] made observations on his own children, all boys, at the age of 2, 4, 10 and 15 years. The oldest and youngest were some-what, the other two very much under weight. Contrary to general experience, the calories per kilo reported to be taken by these children were low. We can hardly resist the inference that, if the diet at the time of observation was typical of that usually taken, these children were below weight because underfed.

Camerer[14] made extensive observations of the amount of food taken by his own children, four girls and one boy, at different times extending over a period of years. In the observations made at ages under 7 years the calories per kilo taken by both sexes were about the usual for those ages. In the later observations, however, the calories per kilo were very low and diminished rapidly with increasing age, so that in the observations made at ages over 15 years the calories per kilo taken by both sexes were only a little above the basal requirements as determined by Benedict and Talbot. Camerer's girls were on the average much below the average weight for age at the time of observa-tion and during the early years the boy was somewhat under weight. Accordingly a high caloric intake would have been expected. The fact that, on the contrary, the calories per kilo taken by these children were very low suggests, as we have said in regard to Uffelmand's children, that the under weight was possibly due to under feeding.

Baginsky[15] has given values obtained from a study of a number of children convalescent from various forms of acute disease and with one exception much under weight. As might be anticipated, his values are for the most part high.

E. H. Starling,[16] quotes Carl Tigerstedt, who made forty-seven observations on children ranging from 4 to 14 years of age. The weights unfortunately are not given, only the calories per kilo. The striking thing in these observations is the wide range of calories taken.

13. Uffelmann, J.: Hygiene des Kindes, 1881.
14. Camerer: Stoffwechsel des Kindes, 1896.
15. Baginsky, A.: Arch. f. Kinderh. **23**:119, 1897, and **16**:398, 1893.
16. Starling, E. H.: The Feeding of Nations, 1919.

For example, in four observations on children in their fifth year the range was from 73 to 114 kilos; in five observations for the seventh year, from 71 to 102; nine observations for the twelfth year, from 44 to 89; seven observations for the fourteenth year, from 43 to 82 calories per kilo. Starling calls attention to the dangers involved in basing estimates for food requirements on averages, especially when these include only a small number of observations.

In marked contrast to the values found by most of the authors mentioned above are those of Gephart[17] whose observations were made in quite a different manner. His method was that employed by other writers in studying the actual amount of food consumed by soldiers in camps, and seems to be a reliable way of determining the average number of calories taken. Gephart studied the diet of about 350 boys in a large boarding school (St. Paul's, Concord, N. H.) in the following manner: He first calculated the caloric value of the entire amount of food purchased during the period of observation, the entire school year. From this he subtracted the values obtained by analyzing at various times the garbage and the waste. The remainder he divided by the total number of meals served, and thus obtained an average caloric value per meal for the school. In addition to the meals which were provided by the school, the boys were accustomed to buy from a confectioner's shop considerable extra nourishment—sweets, chocolate, cakes, etc. The total amount purchased during the period was known and was apportioned by Gephart among the boys of the whole school. The calories furnished by this additional food were found to be about one-eighth of the total daily consumption.

The average daily caloric intake was found to be about 5,000 calories per boy, and all but the group of the oldest boys took over 100 calories per kilo. The boys were divided into three groups, the lower, the middle and the upper school. In Table 2 are given the ages, average net weight, calories per kilo furnished by the school, additional calories purchased at the confectioner's, average total calories per kilo and average daily total calories for each group.

While the conclusions which might be drawn from these observations may perhaps be open to question, still, the results show what

17. Gephart, F. C.: Boston M. & S. J. **176**:17, 1917.

amount of food is actually taken by the average American school boy at the ages studied, under the special conditions represented by these observations.   It may be argued that these conditions were not average but somewhat exceptional.   The boys were living in a rigorous climate; they were taking a great deal of active out-of-door exercise and they were at an age when growth is most rapid.   Furthermore, the well-known disposition of boys of these ages to stuff themselves with food apparently beyond their actual needs must also be taken into account.   Still when due allowance has been made for all these conditions, the fact remains that the enormous appetite of active, growing boys represents a physiological need which in the past has not been given sufficient consideration.

TABLE 2.

Summary of Gephart's Observations on Boys at St. Paul's School.

School	Average Age in Years	Average Weight		Average Calories per Kilo			Average Total Daily Calories
		Kilos	Pounds	School	Food Shop	Total	
Lower.........	13 5	43 6	96	98	15	113	4,949
Middle........	14 5	50 8	112	88	13	101	5,126
Upper	16 1	60 6	133	71	11	82	4,997

Studies made on such large groups are likely to give results much nearer the truth than observations made on a few individuals or the children of one family, no matter how carefully these observations have been made.   The results of the German observations which we have quoted would lead one to allow too little food for children during the active growing period.

The Department of Agriculture has made many observations on the amount of food taken by families, including children, but has not determined experimentally what proportion of the food was taken by each child.   They have apportioned the diets theoretically according to a commonly used system of coefficients; for example, assuming that if a man takes one portion, a woman takes 0.8, a boy of 12 takes 0.8, a boy of 8 takes 0.7, etc.

Schedules Proposed by Various Authors.—Several authors have proposed complete schedules of theoretic caloric requirements from

infancy to adult life. These have been based either on their own observations or on those of others. Most of these observations we have just discussed.

Among the German observers, Camerer's schedule has been most often quoted as a standard. His suggested allowance for boys is 89 calories per kilo at the age of one year and diminishes to 75 calories at 4 years. For the age of 5 years the allowance is increased to 84 calories. From that age the allowance decreases rapidly and steadily to adult life. His values after 6 years are low, and after 9 years extremely so. After the twelfth year his allowance is but little above the basal requirements as shown by Benedict and Talbot.

Steffen[18] offers a schedule for children up to six years of age and allows over 100 calories per kilo through this entire period.

Uffelmann has proposed a schedule for the first five years of life. His values are rather low, ranging from 88 calories per kilo at one year to 68 calories at five years.

Gillett and Sherman, after giving due consideration to the observations which have been published and which are summarized by Gillett, present a table of suggested values for total daily calories for children of both sexes throughout the entire period of growth. They allow a considerable range of variation. Their allowance diminishes gradually to 68 calories per kilo at 9 years and is maintained at that figure up to the age of 13, dropping to 65 at 14 years. After this age the average allowance is rapidly decreased to 55 calories per kilo at 16 years. This decrease in calories per kilo during the period of most rapid growth after the age of 13 seems to us injudicious.

Lusk has recently published estimates for total calories. As already mentioned, he makes a very large allowance for activity and gives three curves—for the quiet child, the active child and the very active child. He allows the same percentage increase in total calories, for increased activity, quite irrespective of age and weight. That is, his values for the active child are about one and one-third times those for the quiet child at all ages, while the values for the very active child are about twice those for the quiet child at all ages. Accordingly, his estimates for total calories for the very active child and even for

18. Steffen, W.: Jahrb. f. Kinderh. 46:332, 1898.

the child of moderate activity, are extraordinarily high for the early years, for example, 193 and 129 calories per kilo, respectively, for a boy of 2 years. The diminution in calories per kilo with increasing years is rapid according to all three of his curves. His values for the quiet child after the age of 13 are very little above the calories necessary for basal metabolism and for normal growth. No allowance is left for activity, which of course can never be reduced to zero.

Gillett and Sherman and also Camerer give schedules for girls as well as for boys. According to both the allowance for girls is considerably lower than that for boys, and from the twelfth year the allowance exceeds only by very little the needs for basal metabolism and growth.

Proposed Schedules for Calories per Kilo.

In the light of our own observations and those of others, the theoretical schedules for calories per kilo of body weight shown in Charts 1 and 2 and Tables 3 and 4 are suggested. The charts show the curves and the tables the actual figures, on which the curves are based, for the different factors which make up the total calories per kilo. The tables show also the percentage allowance for each of the different factors.

The basal requirement adopted is that of Benedict and Talbot. This, it will be seen, after reaching a maximum at about the ninth month, falls steadily through the entire period of growth to adult life. The basal requirement per kilo for girls is lower than that for boys up to the age of 11 years, after which it is higher than that for boys until the completion of growth.

The growth allowance has been calculated from the average normal rate of growth, which has been well established. The calories allowed for growth fall steadily from the first year to the sixth year, then remain practically constant up to eleven years for girls and thirteen years for boys, when a marked increase takes place. This increased need is evident for about three years with both sexes, after which the growth needs rapidly diminish to zero.

The needs for basal metabolism and for growth, though subject to considerable individual variation, are, as averages, practically irreducible.

TABLE 3.

Suggested Calories per Kilo for Boys.

Weight, Kilos	Calories per Kilo					Per Cent. of Calories for				Total Calories Daily
	Basal	Growth	Activity	Excreta	Total	Basal	Growth	Activity	Excreta	
3	46	56	8	10	120	38	47	7	8	360
4	50	52	8	10	120	42	43	7	8	480
5	54	46	8	10	118	46	39	7	8	590
6	56	38	10	10	114	49	33	9	9	685
7	57	32	11	10	110	52	29	10	9	770
8	56	28	12	10	106	53	26	11	10	850
9	55	25	12	10	102	54	24	12	10	920
10	54	22	13	10	99	55	22	13	10	990
11	53	19	14	10	96	55	20	15	10	1,060
12	52	16	16	9	93	56	17	17	10	1,120
13	51	14	17	9	91	56	16	18	10	1,180
14	50	13	17	9	89	56	15	19	10	1,240
15	49	12	17	9	87	56	14	20	10	1,300
16	48	10	18	9	85	56	12	22	10	1:360
17	47	9	19	8	83	56	11	23	10	1,410
18	46	8	20	8	82	56	10	24	10	1,470
19	45	7	21	8	81	56	9	25	10	1,540
20	44	7	21	8	80	55	9	26	10	1,600
22	42	7	23	8	80	53	9	28	10	1,760
24	41	8	23	8	80	51	10	29	10	1,920
26	39	8	25	8	80	49	8	31	10	2,080
28	38	7	27	8	80	47	9	34	10	2,240
30	36	7	29	8	80	45	9	36	10	2,400
33	35	7	30	8	80	44	9	37	10	2,640
36	33	8	31	8	80	41	10	39	10	2,880
39	32	9	31	8	80	40	11	39	10	3,120
42	31	10	31	8	80	39	12	39	10	3:360
45	30	11	31	8	80	38	13	39	10	3,600
48	30	11	31	8	80	38	13	39	10	3,840
51	29	11	31	8	79	38	13	39	10	4,030
54	29	8	31	8	76	38	11	41	10	4,100
57	28	5	30	7	70	40	7	43	11	3,990
60	27	2	27	7	62	43	3	44	10	3,720
68 (Adult)	25	0	18	5	48	52	0	38	10	3,265

The allowance for activity is admitted to be hypothetical. For reasons previously given, this has been increased steadily from the second to the thirteenth year for boys and to the twelfth year for girls. Whether the allowance for activity should be further increased

TABLE 4.

Suggested Calories per Kilo for Girls.

Weight, Kilos	Calories per Kilo					Per Cent of Calories for				Total Calories Daily
	Basal	Growth	Activity	Excreta	Total	Basal	Growth	Activity	Excreta	
3	51	51	8	10	120	43	43	6	8	360
4	53	49	8	10	120	45	41	6	8	480
5	55	45	8	10	118	47	38	7	8	590
6	56	38	10	10	114	49	33	9	9	685
7	56	33	11	10	110	51	30	10	9	770
8	57	27	12	10	106	54	25	11	10	850
9	56	24	12	10	102	55	23	12	10	920
10	55	21	13	10	99	56	21	13	10	990
11	53	19	14	10	96	55	20	15	10	1,060
12	51	17	16	9	93	55	18	17	10	1,120
13	49	16	16	9	90	54	18	18	10	1,170
14	47	14	17	9	87	54	16	20	10	1,220
15	45	12	19	8	84	54	14	22	10	1,260
16	44	11	19	8	82	54	13	23	10	1,310
17	43	10	19	8	80	54	12	24	10	1,360
18	42	9	19	8	78	54	12	24	10	1,400
19	41	8	20	8	77	53	11	26	10	1,460
20	40	7	21	8	76	53	9	28	10	1,520
22	38	8	22	8	76	50	11	29	10	1,670
24	37	8	23	8	76	49	11	30	10	1,820
26	36	8	24	8	76	48	10	32	10	1,980
28	36	8	25	8	77	47	10	33	10	2,155
30	36	9	26	8	79	46	11	33	10	2,370
33	35	10	27	8	80	44	12	34	10	2,640
36	35	10	27	8	80	44	12	34	10	2,880
39	35	10	27	8	80	44	12	34	10	3,120
42	34	9	27	8	78	44	11	35	10	3,275
45	34	7	26	7	74	46	9	35	10	3,330
48	33	5	22	7	67	49	8	33	10	3,215
51	32	3	21	6	62	51	5	34	10	3,160
54	31	0	18	5	54	57	0	33	10	2,915
60 (Adult)	25	0	15	4	44	57	0	33	10	2,640

during the period of most rapid growth (in girls from the twelfth to the fourteenth or fifteenth year and in boys from the thirteenth to the seventeenth) is somewhat doubtful. It is a matter of common observation that while the body is increasing so rapidly in size as it does at this time, "the lazy age," there is frequently seen in both sexes

a growing disinclination to active muscular exertion, which is usually accompanied by a corresponding disinclination to mental activity, sometimes amounting almost to mental lethargy. A very considerable stimulus may be needed to voluntary effort, both mental and physical. This is not surprising; it is rather to be expected and may be considered physiologic. The opinion is not only an impression from our own observations but is confirmed by the head masters and athletic directors of six large schools for boys with whom we have discussed the question. For the reasons above given we have felt that no increase in the caloric allowance per kilo for activity should be made during these years of most active growth; possibly even a slight reduction should be considered.

The number of calories we have allowed for loss in the excreta is 10 per cent. of the total after the first year of life, during which period a somewhat smaller proportion should be allowed, certainly for nursing infants.

With the values proposed for the different factors just discussed the total calories per kilo are for both sexes about 120 during the early part of the first year, diminishing to 100 at one year, and reaching 93 calories per kilo at 2 years. After the second year there is an appreciable difference between the total calories per kilo allowed for boys and for girls, owing largely to the difference in their basal requirement, that of girls being, from this time until the end of the tenth year, several calories lower than that of boys. The calories per kilo allowed for both sexes slowly fall, largely because of the slowing up of the growth rate. We have estimated that at 6 years the total for boys should be 80 calories per kilo and for girls 76 calories per kilo. This value we have continued for boys up to about the sixteenth year. For girls the calories per kilo have been increased from 76 to 80 during the eleventh year, since there is at this time an increased growth need and since the basal needs for this and the next few years are nearly uniform. This higher value is continued for girls to about the fourteenth year. After the sixteenth year in boys and the fourteenth year in girls, since there is a rapid decline in growth needs and possibly some reduction in activity, the total calories per kilo have been rapidly reduced to adult standard—about 48 for males and 44 for females.

The chief difference between our schedule for total calories per kilo and others which have been proposed, is that in our schedule the total calories per kilo are nearly uniform from the age of six to the end of the period of rapid growth.

In the practical application of these suggested values it must be borne in mind that each component part of the caloric requirement is subject to considerable individual variation. The curve suggested aims only to give averages. The calories actually given must vary to fit the individual needs. Activity is of course the most obvious variant. An extremely active child will undqubtedly utilize more calories than the average which we have allowed, while the needs of a quiet child may be supplied by somewhat less than our suggested values.

Another cause for variation in the caloric requirement for the individual is the relation of weight to height and age. Our observations and those of others show that the underweight child will take and utilize more calories per kilo than the child of average weight; while, as might be expected, one who is overweight does not take and does not need the average number of calories per kilo. The fact must be taken into account in making the caloric allowance for an individual child.

It seems reasonable to allow for the younger children·a normal range of five calories per kilo above and below the average given, and to allow the older children a range of eight to ten calories above and below the average. For instance, the normal range allowed for a boy of 2 years might be from 88 to 98 calories per kilo, for a boy of six from 72 to 88 calories and for one of 15 a range from 70 to 90 calories per kilo. When activity is excessive, however, an increase of 20 or even 30 calories above the average may be well utilized.

Comparison of Schedules Proposed.—In order to show the difference between our suggested schedule for calories per kilo and others which have been proposed, Table 5 and Chart 4 are given. The table shows the calories per kilo proposed by various authors for both boys and girls at different ages. This table brings out clearly the points already mentioned in discussing the schedules offered. The only schedule which allows more calories per kilo throughout the entire range of years than does ours is that of Lusk for the very active child. During the early years this schedule shows about

twice as many calories per kilo as we have considered sufficient. The difference diminishes after the age of seven, until at the age of fifteen his values are but little above ours. Lusk's estimate for the child of moderate activity exceeds ours up to the age of twelve, after which his values fall considerably below ours. The other complete schedules, those of Gillett and Camerer, are much below ours after the age of seven or eight. The two schedules for girls show values very much below ours after the age of six years.

TABLE 5.

Comparison of Schedules for Total Calories per Kilo.

Author	Age, Years															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Boys:																
Holt and Fales	100	93	88	84	82	80	80	80	80	80	80	80	80	80	80	75
Gillett	105	90	83	79	77	75	73	71	68	67	68	69	68	65	60	55
Lusk, quiet child	79	97	90	88	79	78	79	73	70	66	66	62	54	48	45	
Lusk, active child	.	129	117	116	105	105	105	97	93	88	83	82	73	66	62	
Lusk, very active child		193	177	175	158	158	159	146	139	132	124	123	109	98	93	
Camerer	89	80	78	75	84	77	72	67	63	58	55	52	49	47	45	42
Uffelmann	88	80	73	71	68											
Steffen	113	99	105	125	114	103										
Girls:																
Holt and Fales	101	94	87	82	78	76	76	76	76	77	80	80	79	74	67	62
Gillett	105	93	83	79	73	71	69	66	64	62	59	55	51	48	47	46
Camerer	89	80	78	75	70	67	64	62	60	58	55	51	47	43	41	39

On Chart 4 the basal metabolism and the growth needs are indicated by the heavily and lightly shaded areas respectively. As we have said, these are, as averages, practically irreducible. The various curves shown in the chart were obtained by plotting the values offered in the various schedules after subtracting the ten per cent. of the total calories which is lost in the excreta. Therefore, the space between the shaded areas and the various lines represents the allowance for activity according to the different authors. The great difference is at once apparent. The curves representing total calories per kilo would be obtained by raising each of the curves shown by the addition to the values plotted of the ten per cent. allowance for loss in the excreta.

The curve based on Camerer's values shows very little allowance for activity except between the ages of 4 and 8 years and after the age of 13 years practically none at all.
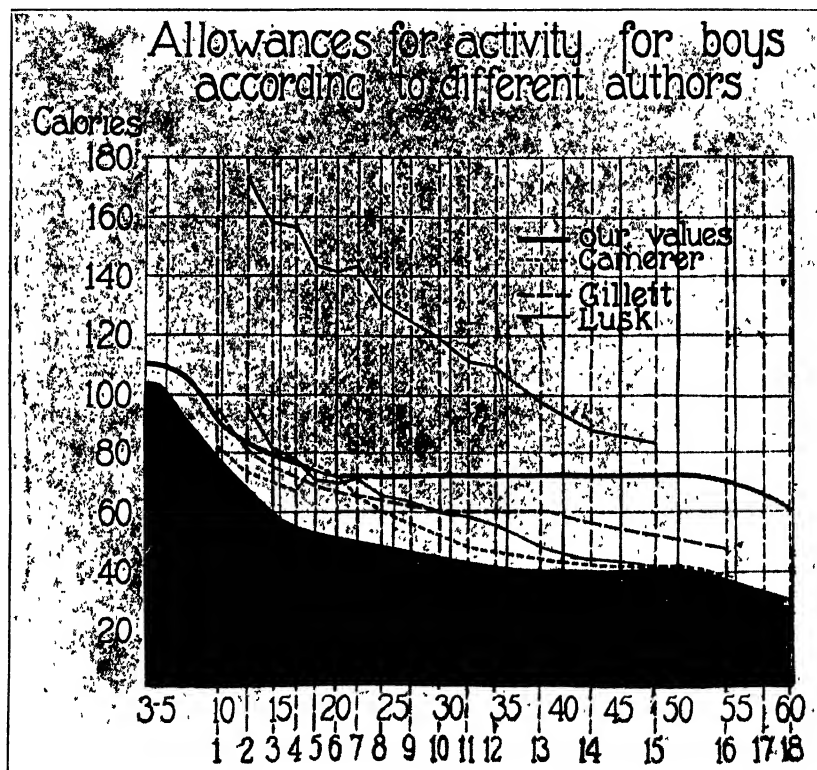
FIG. 4.—The heavily shaded area represents allowance in calories per kilo for basal metabolism; the lighter shaded area, the allowance for growth; both areas are according to our schedule. The spaces between the shaded areas and the lines above show what remains for activity according to the estimates of different authors. The solid vertical lines indicate weight; the broken lines, approximate weight at each year of age.

The curve based on the average values offered by Gillett and Sherman does not differ greatly from our own up to the age of 7 years. From 7 to 14 years it is nearly parallel with ours, but somewhat lower. After 14 years their curve falls rapidly.   In our judgment they have allowed far too little for activity and growth during adolescence. As previously stated, they allow a fairly wide individual variation

in total calories, but even if their maximum allowance is taken, the calories per kilo after the age of 14 are only about five calories higher than the values shown on the chart, and therefore, still about fifteen calories per kilo lower than the values we have suggested.

The curve based on Lusk's values for the quiet child is almost identical with our own from 2 to 7 years. After this age his allowance for activity diminishes very rapidly and becomes practically zero at fourteen years. He gives a schedule, not represented here, for the child of moderate activity which, when plotted, is represented by a curve almost parallel to the one just discussed but about one-third higher. His curve for the very active child seems to give an extreme allowance for activity, certainly for the first ten or twelve years of life. At the age of 15 his allowance is not far above our own.

Total Daily Calories.—Charts 5 and 6 and Table 6 give for boys and girls the average values for total daily calories based upon the values for calories per kilo just proposed by us. The curves shown on the chart are plotted on the basis of years of age, using the average normal weights at the different ages. The charts show not only the total daily calories but also the components—basal needs, growth needs, allowance for activity and for loss in the excreta—which together make up the total.

The daily calories allowed for the boys and girls are about 950 at one year. They increase with succeeding years for both sexes, the increase for the boys being a little more rapid than for the girls until the age of 12 is reached. From 12 to 14 the total calories for the girls exceed those for boys. After fourteen, the allowance for boys is considerably greater than that for girls. The highest values for daily calories are 3,330 for girls at the age of 14 and 4,100 for boys, at the age of 16. After these maximum figures are reached the values drop very rapidly to adult standards for moderate activity,—about 2,640 for women and 3,265 for men.

These charts show the provision for the increase in the growth needs during the period of adolescence. The growth need, calculated according to Rubner's formula, reaches for girls 380 calories daily during the thirteenth year, and for boys about 500 calories daily during the sixteenth year. It is this greatly increased need of calories for normal growth at this period that has not been sufficiently taken
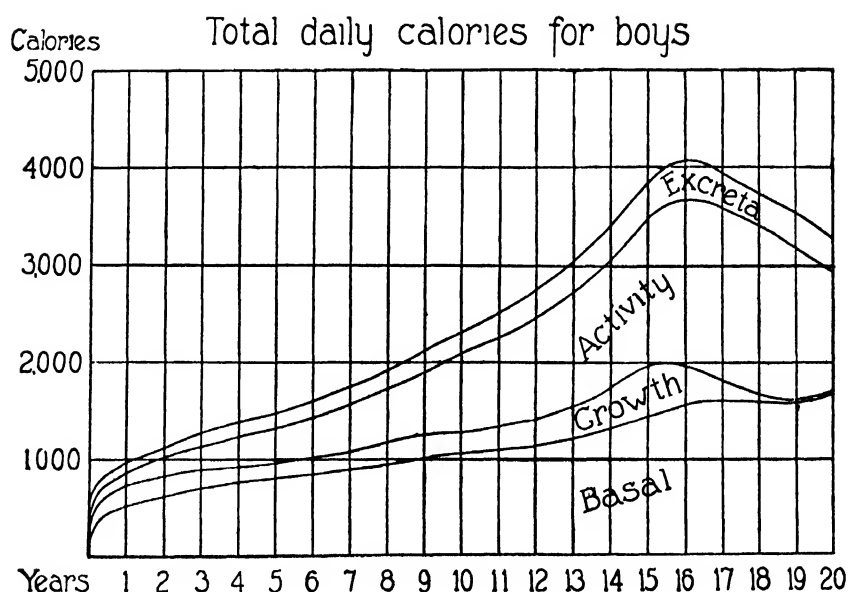
FIG. 5.—The distance between the base and the upper line shows the allowance for total daily calories according to age, from birth to adult life.   The spaces between the various lines, from the base line upward, indicate the allowance for the different factors which make up the total, namely, for basal requirement, growth, activity and loss in excreta.
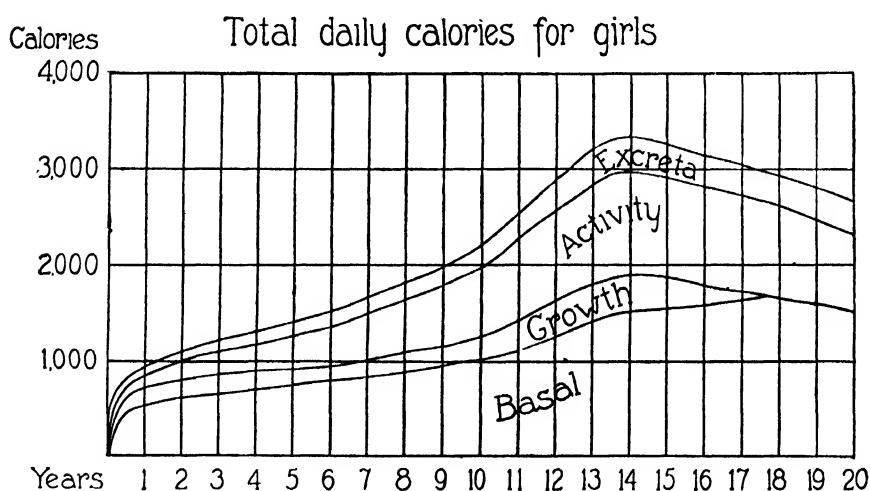
FIG. 6.—The curved lines and spaces have the same significance as in Chart 5.

into account by many authors in estimating the total daily calories required by the growing child.

The large allowance for activity is of course the most debatable factor in our estimate. Some may think that this allowance is excessive but the more recent observations, particularly those of Gephart, seem to justify our position. Our values for the total daily caloric allowance for boys of the age of those studied by Gephart are still

TABLE 6.

Suggested Total Daily Calories.

Age Years	Boys					Girls				
	Average Weight		Calories per		Total Daily Calories	Average Weight		Calories per		Total Daily Calories
	Kilos	Pounds	Kilo	Pound		Kilos	Pounds	Kilo	Pound	
1	9 5	22	100	45	950	9 3	21	101	45	940
2	12.2	27	93	42	1,135	11 8	26	94	43	1,110
3	14 5	32	88	40	1,275	14 1	31	87	40	1,230
4	16 4	36	84	38	1,380	15 9	35	82	37	1,300
5	18.2	40	82	37	1,490	18 2	40	78	36	1,410
6	20 0	44	80	36	1,600	20 0	44	76	34	1,520
7	21.8	48	80	36	1,745	21 8	48	76	34	1,660
8	24 0	53	80	36	1,920	23 9	53	76	34	1,815
9	26 4	58	80	36	2,110	26 2	58	76	34	1,990
10	29.1	64	80	36	2,330	28 5	63	77	35	2,195
11	31 4	69	80	36	2,510	31 5	69	80	36	2,520
12	34 2	75	80	36	2,735	35 8	79	80	36	2,864
13	38 0	84	80	36	3,040	40 6	89	79	36	3,210
14	42 5	94	80	36	3,400	45 0	99	74	34	3,330
15	48 2	106	80	36	3,855	48 3	106	67	30	3,235
16	54.5	120	75	34	4,090	51 0	112	62	28	3,160
17	57 5	127	69	31	3,945	52 6	116	58	26	3,060
18	59 8	132	62	28	3,730	52 8	117	56	25	2,950
Adult	68 0	150	48	22	3,265	60 0	132	44	20	2,640

much below the calories which he found to be actually taken by the boys he observed. However, as we have stated, these boys were living under rather exceptional conditions and the calories taken by them can hardly be used as a basis for estimating normal averages.

According to our schedule the allowance for activity varies from 6 per cent. of the total calories during the first year to a maximum of 44 per cent. of the total calories during the period of adolescence This does not seem excessive for this active period.

It is a common observation and an undoubtedly true one, that during adolescence the average boy or girl takes more food than the average adult man or woman. Unquestionably, this represents a real physiologic need. It is only by assuming such values for calories per kilo as we have proposed that one can reach a total daily caloric intake which will be in accord with these conditions.

The several authors who have given complete schedules for food requirements steadily increase the total daily calories with age through childhood and adolescence up to the standard allowance for adults. No one has up to the present proposed a schedule according to which the values for total daily calories exceed adult standards during adolescence and drop to adult standards with the completion of growth. This seems to us to be absolutely necessary. Unless this is done, the increasing growth needs must be deducted from the allowance for activity, leaving for the latter a very small remainder; or, on the other hand, if activity is maintained, growth must suffer. The modern child, boy or girl, is usually an active person during adolescence. If in school, he is required by school routine to take systematic exercise. If he has left school and entered industrial life he may be continually compelled to expend physical energy. In view of these facts it is evident that the food taken by perhaps the majority of boys of the poorer classes who are engaged in active industrial occupations during this period of rapid growth, is totally inadequate. An arrest of growth is the almost inevitable consequence of forced activity on a limited ration.

Original Observations.

Because there have been so few observations reported in the literature which give the calories taken by normal children under average conditions and because of the objections, already discussed, to drawing conclusions from a small number of observations, we have endeavored to collect a sufficient number of individual diet records to warrant some conclusions. This has proven to be a laborious and difficult task. We have at present, however, obtained dietary records from over one hundred selected children of both sexes from 1 to 16 years of age, who were healthy, well cared for, and normal as to digestion. They were almost all children in private families, living in excellent

surroundings. The parents were intelligent people, interested in the purpose of our experiment, and willing to cooperate. The children were supposedly intelligently fed and it can be assumed that they were receiving diets fairly typical of those usually taken by well children.

A report of the net weight, height, activity, appetite, general condition, etc., was obtained, together with a record of the exact amount of each item of food taken by the child for four consecutive days. From these data the caloric value of the average daily diet was calculated. Not only was the value for the total calories obtained, but also the distribution of the calories as fat, carbohydrate and protein, which, together with other facts brought out, will be discussed in succeeding papers. Established caloric values of the common articles of food have been compiled in a convenient form by Locke.[19] These have been for the most part used. Other authors whose publications we have found valuable for reference in this study are Leach,[20] Sherman,[21] Lusk[22] and Jordan.[23] The values of a number of articles were calculated from the results of analyses made in our own laboratory.

This method of estimating the calories taken by children is of course not absolutely accurate but it undoubtedly gives a very close approximation. While there are slight variations from the usual caloric values of the food taken owing to differences in the methods of preparing food, it is probable that these errors tend to balance each other and that the net result is approximately accurate. We feel certain that in no case did the child receive less food than was reported. It is not unlikely that in some cases a child actually took more food than was reported, especially the older children whose diet is not so carefully supervised and who are more likely to eat between meals without the knowledge of the parent. If there is, therefore, an appreciable error in the estimation of the calories, it is that the calculated amount is somewhat low.

19. Locke, E. A.: Food Values, 1917.
20. Leach, A. E.: Food Inspection and Analysis, 1920.
21. Sherman, H. C.: Chemistry of Food and Nutrition, 1919.
22. Lusk, G.: The Science of Nutrition, 1919.
23. Jordan, W. H.: The Principles of Human Nutrition, 1919.

The largest number of the records were of children under 11 years of age. There are from five to twelve observations for each year up to the age of 11. Beyond this age the number of observations for each year is too small to warrant definite conclusions.

In Chart 7 are shown the total calories taken by these children arranged according to age. The curves shown are those based upon the schedules proposed by us for average daily calories at different ages. The curve for boys is indicated by the solid line, that for girls by the broken line. The individual observations for boys are shown by the dots, those for girls by circles.

This chart shows, as was to be expected, a wide variation in individual observations. However, the observations mostly fall near the curves and up to the age of 11 the average would fall very near the curves. Beyond that age there are as yet too few observations to be conclusive. However, those made on older boys are very close to the proposed curve.

Some of the observations which vary most widely from the average curves deserve special mention. There were eight children in the series whose total calories taken amounted to over one-third more than the theoretical average requirement, but every one of these children was reported to be exceedingly active. There were only two children, both girls, whose total calories were more than one-third less than the theoretical average requirement, but these girls were both very large for their ages, in fact, had nearly attained adult stature and accordingly did not need the number of calories for growth usual for that age.

On the whole, it would seem that the curves suggested are approximately correct for the average caloric requirements up to the age of 11 years. We feel confident that a larger number of observations for the later years will verify the estimates for those years also.

When the individual observations are considered on a per kilo basis and compared with the proposed curves for calories per kilo, the individual observations show wide variation, but practically all the high values for calories per kilo were found in case of very active children or those who were very much under weight for their height or undersize for their age. The values which were found to be much
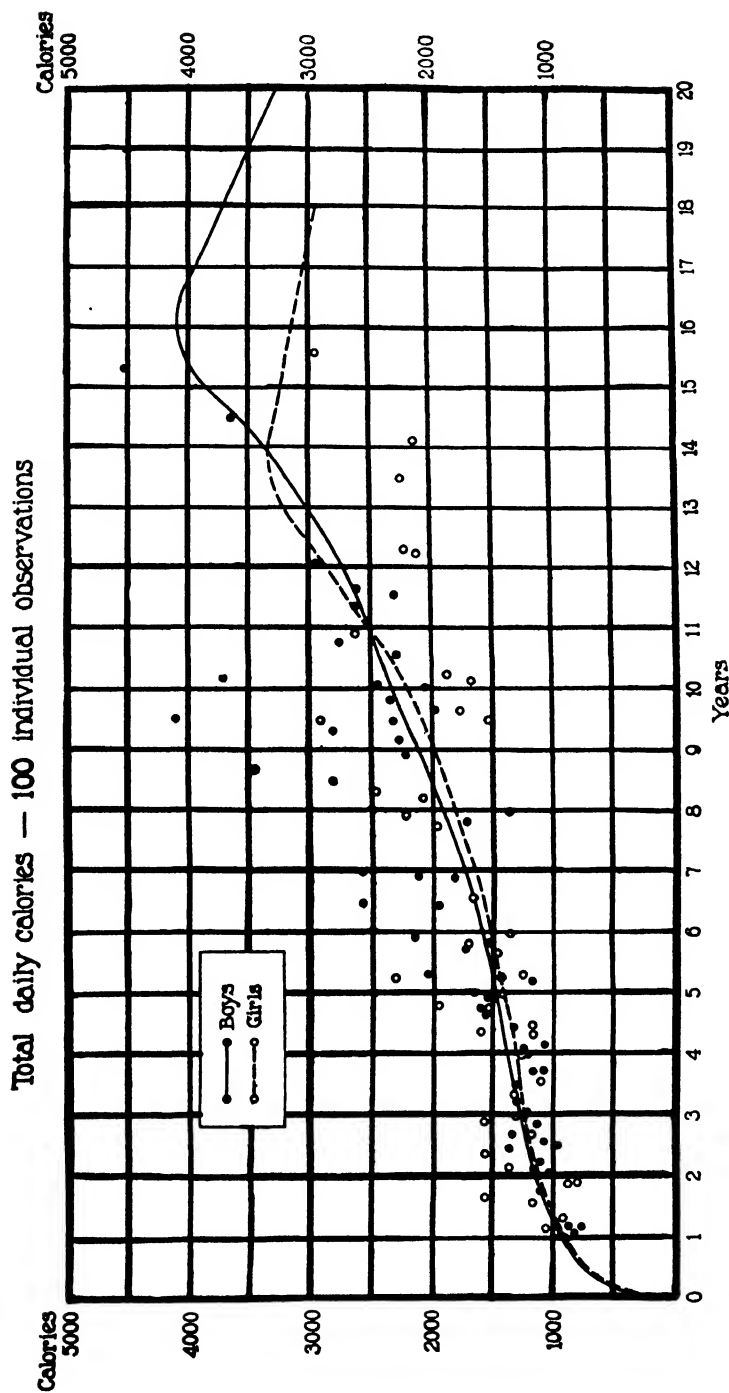
FIG. 7.—Curved lines represent suggested total daily calories according to age, the solid line the curve for boys, the broken line for girls. The dots represent the calories taken in individual observations on boys, the circles those taken by girls.

lower than the average shown by the curves were mostly in case of quiet children or those who were either large for their age or over weight for their height.

SUMMARY.

1. In calculating the total caloric requirements of children there must be considered separately the component parts of which the total is made up. These are the requirements for basal metabolism, for growth, for muscular activity and the food values lost in the excreta.

2. The basal requirements, which have been determined by Benedict and Talbot, are highest per kilo at about nine months and steadily fall from this time up to adult life.

3. The food value normally lost in the excreta is a nearly uniform proportion of the intake, about 10 per cent., for all ages after infancy.

4. The requirements for growth are greatest during the period when growth is most active, namely, during the first year of life and during adolescence. They are nearly uniform from the fourth to the tenth or eleventh years.

5. The average for three factors—basal, growth requirements and food values lost in excreta—are nearly uniform for children of the same weight living under similar conditions.

6. The requirement for activity is the only factor which varies widely with different individuals.

7. The great differences in the calculations of different writers who have estimated theoretical values for total calories per kilo for children are in part due to the fact that they have not sufficiently considered the different component parts which make up the total.

8. The average caloric requirement of children according to our observations is about 100 calories per kilo at an age of 1 year (about 9.5 kilos). For boys it falls to about 80 calories at 6 years (about 20 kilos) and remains practically constant at this value up to the age of 15 years, the increasing requirements for activity being met by the reduction in basal requirements per kilo. After a weight of 50 kilos (about 15 years) is reached, the calories per kilo can rapidly be reduced to adult standards, about 48 calories per kilo. The requirement for girls falls to 76 calories per kilo at 6 years (about 20 kilos), continues at this value until the tenth year. During the tenth year

it rises because the basal requirement is nearly constant while there is an increase in needs for growth and activity. The requirement remains at 80 calories per kilo until growth is complete, then falls rapidly to adult standards, about 44 calories per kilo.

9. In our calculation we have allowed a much higher value for calories per kilo during adolescence than have others. This seems to be absolutely essential, because of the increased growth needs at this time and the large requirement for muscular activity.

10. According to our allowance the total daily caloric requirement of children of both sexes during adolescence exceeds by nearly 1,000 calories the requirements of the adult man or woman of moderate activity.

11. Children who are under weight require more calories per kilo than those who are of average weight for their age. Children who are over weight require fewer calories per kilo than those of average weight.

12. The schedule here proposed is a tentative one and is based on present knowledge, which is in many respects incomplete. There are a number of points which must be studied more fully before definite standards can be established.

For the many reasons which we have discussed, it seems the right procedure to allow for children during the period of adolescence more calories than the adult ration and, as growth needs end to diminish the caloric allowance to the adult standard. We quite agree with Benedict's recent statement, "It is still, however, the best practice to give a most liberal diet to children, since the greater part of the evidence on under weight indicates that children usually receive too little rather than too much food."

THE CARBON DIOXIDE CARRIERS OF THE BLOOD.

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The arterial blood of man normally contains about 50 volumes per cent of carbon dioxide, the venous about 55 to 60. A pure solution containing 50 volumes per cent of carbon dioxide would have a hydrogen ion concentration of 3.1×10^{-5}. Yet in the blood the hydrogen ion concentration is only $\frac{1}{1000}$ as great, and the difference between arterial and venous blood is so slight as to be barely within the limits of measurement by our most sensitive methods. The purpose of the present paper is to discuss the chemical mechanism by which blood is enabled to carry its allotted load of carbon dioxide, and nevertheless maintain its hydrogen ion concentration at such a low and remarkably constant level.

Briefly stated, the organism utilizes two properties of certain constituents to set the blood pH[1] at the physiological point, and to maintain it near that point despite variations in CO_2 content such as occur in the change from venous to arterial blood. One of these properties is the buffer action of the bicarbonate and the proteins in the plasma, and of bicarbonate, proteins, and phosphates in the cells. The other property is the change from a relatively weak acid to a relatively strong one which hemoglobin undergoes when it changes from the reduced to the oxidized form. We shall discuss these factors sepa-

[1] In speaking of reaction or hydrogen ion concentration we shall in this paper express data in the terms of pH introduced by Soerensen (1912) and generally adopted because of their convenience. The pH at a given reaction is the negative logarithm of the normality of the hydrogen ion concentration. The hydrogen ion concentration of a 0.01 N HCl solution, for example, is approximately 0.01 N, since the dissociation of HCl into H$^+$ and Cl' is practically complete. 0.01 is 10^{-2}. The logarithm of 10^{-2} is of course -2. The pH of 0.01 N HCl solution is therefore 2. The pH of water is almost exactly 7, which therefore represents the neutral reaction. A pH greater than 7 indicates an alkaline reaction, less than 7 an acid reaction. The reaction of normal blood at pH 7.35 is therefore slightly alkaline.

rately, and then attempt to outline the manner in which they combine to enable the organism to meet both ordinary and extraordinary demands upon it for transport of carbon dioxide, and still maintain almost absolute constancy of hydrogen ion concentration in the blood.

Forms in which Carbon Dioxide is Carried in the Blood.

There are four forms which CO_2 may assume in a solution, viz., dissolved anhydrous carbon dioxide, carbonic acid, bicarbonate, and carbonate. Using B to indicate a monovalent base, such as Na or K, these forms are indicated by the molecular formulas, CO_2, H_2CO_3, $BHCO_3$, and B_2CO_3 respectively. Concerning the equilibrium between the first two forms, CO_2 and H_2CO_3, we are uncertain. We can, however, assume that, if all the CO_2 in pure water solution is not changed to H_2CO_3, at least a constant proportion of it is. The reaction for the change is

$$CO_2 + H_2O = H_2CO_3.$$

From the mass law the conditions for equilibrium are

(Concentration of CO_2) $\times$ (Concentration of H_2O) $=$ Constant $\times$ (Concentration of H_2CO_3).

The H_2O concentration is constant, however, so the above equation becomes:

Concentration of CO_2 $=$ Constant $\times$ Concentration of H_2CO_3.

Since the H_2CO_3, therefore, either represents all the CO_2 not bound by bases, or represents a constant portion of it, it is customary in the study of equilibrium to assume that all of the CO_2 is in the form H_2CO_3. All calculations of the acid dissociation constant of H_2CO_3 have been based on this assumption, and no errors are introduced if in the use of this constant the same assumption is consistently made. Throughout the present paper, therefore, we shall follow the custom of indicating all the dissolved CO_2 not bound by bases as H_2CO_3.

Of the other two forms, $BHCO_3$ and B_2CO_3, only the bicarbonate can exist in significant quantities in solutions containing as much H_2CO_3 as does the blood. The reaction $B_2CO_3 + H_2CO_3 = 2BHCO_3$ possesses such an equilibrium constant that with physiological concentrations of H_2CO_3 it goes with practical completeness from left to right. This was shown by Bohr (1909):

In a 0.155 per cent sodium carbonate solution (about the average carbonate concentration of plasma) at 38°, and with the physiologically normal carbon dioxide tension of 45 mm., Bohr calculated that 99.5 per cent of the sodium carbonate was in the form of bicarbonate, and confirmed the calculation experimentally within the limit of analytical error. Even at 12 mm. CO_2 tension, which is seldom if ever observed in life except in premortal coma, 98 per cent was in the form of bicarbonate. Consequently one can, for quantitative purposes, regard the bicarbonate of the plasma as synonymous with its entire reserve of alkali in excess of that neutralized by acids other than carbonic.

In considering CO_2 transport by the blood we may therefore limit ourselves to two forms, free CO_2, indicated as H_2CO_3, and bicarbonate, $BHCO_3$. If all the CO_2 of the blood were in the free form as H_2CO_3, the blood would, as shown above, be 1000 times more acid than it is, or can be with compatibility to life. If all the CO_2 were in the form of carbonates, on the other hand, the blood would be hundreds of times too alkaline. The maintenance of the reaction necessary for life requires a definite and constant balance between CO_2 as H_2CO_3 and CO_2 as $BHCO_3$, and it is by means of its mechanism for maintaining such a balance that the organism is able to transport in the blood the great amounts of CO_2 that are continuously carried to the lungs for excretion, and to do so with a barely measurable change in the blood pH.

The Buffers of the Blood.

The Nature of Buffers.

Buffers are salts of either weak bases or weak acids, and are characterized by their ability to enable solutions in which they are present to receive additions of limited amounts of either acid or alkali with much less change of pH than would be caused by the same additions to water, or to a solution containing only salts, such as NaCl, that have no buffer power. All the important buffers of the blood, viz., the bicarbonates, phosphates, and the alkali salts of the proteins, are salts of weak acids. In each buffer, part is present as free acid, part as the salt of a strong base, and the pH of the blood is determined by the relative proportions of buffer salts and free buffer acids respectively.

The alkali salts which constitute the buffers of the blood are in effect reservoirs of alkali, a portion of which they give up to neutralize car-

bonic or any other acid that enters the blood. In this manner act the phosphates, and also the alkali salts of the plasma proteins and of the hemoglobin.

In regard to such buffers two general laws may be stated, the principles underlying both of which may be found in L. J. Henderson's monograph on the regulation of body neutrality (1909).

1. The hydrogen ion concentration of the buffer solution is proportional to the ratio $\dfrac{\text{free acid}}{\text{free salt}}$ or $\dfrac{HA}{BA}$. Examples of such ratios in the blood are $\dfrac{H_2CO_3}{BHCO_3}, \dfrac{BH_2PO_4}{B_2HPO_4}, \dfrac{HHbO}{BHbO}, \dfrac{HHb}{BHb}$. (B is used to indicate any monovalent base, such as Na or K, A to indicate the acid radicle, BHbO the alkali salt of oxyhemoglobin, HHbO the free oxyhemoglobin, BHb and HHb the salt of reduced hemoglobin and the free protein respectively.)

2. A given buffer is most efficient in maintaining constancy of pH (that is, in minimizing the *proportion* by which H^+ is changed by a given addition of acid or alkali) when the ratio $\dfrac{HA}{HB}$ equals 1, and H^+ approximates K, the dissociation constant of the free acid forming one of the buffer pair.

The theoretical demonstration of these two laws is the following:

1. Relationship between pH and the Ratio $\dfrac{HA}{BA}$ or $\dfrac{\text{free buffer acid}}{\text{buffer salt}}$.

This relationship is derived as follows:

Since the reaction of electrolytic dissociation of an acid into H^+ and anion is $HA = H^+ + A'$, it follows from the law of mass action that, at equilibrium,

$$1) \quad K \times HA = H^+ \times A',$$

K being the dissociation constant of the acid. Hence

$$2) \quad H^+ = K \times \frac{H}{A'}$$

But when the buffer mixture is the salt of a very weak acid plus some free acid, only an infinitesimal part of the anion, A', originates from dissociation of the free acid (H_2CO_3 in the concentration present in the blood, for example, is dissociated into H^+ and HCO'_3 only to the

extent of about 1/10,000). Essentially all of the anion originates from dissociation of the salt, BA, into B^+ and A'. Most salts in 0.1 to 0.01 molecular concentration undergo such dissociation to the extent of 60 to 90 per cent of the amount present. If the degree of dissociation be represented by λ, the concentration of anions is $A' = \lambda BA$, and Equation 2 may be written

$$3)\ H^+ = K\frac{HA}{\lambda BA}.$$

Since λ varies to a relatively slight extent over ranges of concentration within such limits as are found in blood constituents, one may state as a close approximation that $\dfrac{K}{\lambda} = K_1$ and

$$4)\ H^+ = K_1\frac{HA}{BA}.$$

In terms of pH, since $pH = -\log H^+$, Equation 4 is

$$5)\ pH = -\log K_1 - \log\frac{HA}{BA}\ \text{or}$$

$$6)\ pH = pK_1 + \log\frac{BA}{HA}.$$

The form represented in Equation 6 was first introduced by Hasselbalch (1916).

Expressing $-\log K_1$ as pK_1 the value of pK_1 for $B_2HPO_4:BH_2PO_4$ mixtures was shown by Soerensen (1912) and by Clark and Lubs (1916) to be approximately 6.8. For $BHCO_3:H_2CO_3$ solutions in the concentrations normally found in blood plasma Hasselbalch (1916) found pK_1 at body temperature to have a value of 6.1.[2] His results indicated in fact that by determination of the ratio $BHCO_3:H_2CO_3$ the pH of a blood sample could be estimated with Equation 6 as accurately as by the standard electrometric method.

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[2] The figure actually given by Hasselbalch is 6.4, because he used the equivalent concentration of H_2CO_3, which is twice the molecular, in calculating the $\dfrac{BHCO_3}{H_2CO_3}$ ratio. When used with the molecular ratio adopted by L. J. Henderson and, so far as we have observed, all other authors except Hasselbalch, the value of pK_1 must, therefore, be reduced by log 2, or 0.3, giving pK_1 the value 6.1.

2. Maximum Efficiency of Buffer Action when $\dfrac{BA}{HA} = 1$, *and* $H^+ = K_1$.

When $\dfrac{BA}{HA} = 1$, H^+ would equal K if BA were completely dissociated. Since only the proportion represented by λ is dissociated, however, $\dfrac{BA}{HA} = 1$ when $H^+ = \dfrac{K}{\lambda} = K_1$. The latter relationships may be demonstrated by substituting 1 for the ratios $\dfrac{HA}{BA}$ and $\dfrac{BA}{HA}$ in equations 3, 4, and 6, above. These then become $H^+ = K\lambda$, $H^+ = K_1$, and $pH = pK_1$, respectively.

The fact that a buffer mixture of a weak acid and its salt has its maximum efficiency, when $\dfrac{BA}{HA} = 1$, in diminishing changes in reaction caused by adding either base or acid, has its basis in the general proposition, that in the ratio $\dfrac{a}{1-a}$ a given change in a produces the least change in the ratio when $a = 0.5$ and the ratio is consequently unity. The relationship is exemplified graphically by a curve expressing as ordinates values of the ratio $\dfrac{BA}{HA}$ as abscissae values of pH. For bicarbonate at approximately normal blood plasma (0.03 M) concentration, the curve indicated by figure 1 is obtained. The curve is calculated from the approximate equation $pH = 6.1 + \log \dfrac{BHCO_3}{H_2CO_3}$, the, for the present purposes, insignificant variations in pK_1 from the value 6.1 being neglected. It is evident from inspection that the curve is steepest in the middle, when H_2CO_3 and $BHCO_3$ are equal (50 per cent of the CO_2 as $BHCO_3$). This fact means that at this point the addition of sufficient acid or alkali to change a given amount of the total CO_2 from $BHCO_3$ to H_2CO_3, or vice versa, causes less shift in pH than at points near either end of the curve, where the ratio $BHCO_3$: H_2CO_3 is much greater or smaller than 1. For example, changing the per cent of CO_2 as $BHCO_3$ from 50 to 60 alters the pH from 6.10 to 6.26, a change of 0.16; while changing the percentage from 85 to 95 raises the pH from 6.85 to 7.40, a change of 0.55. It is evident that

at pH 7.40, the reaction of normal blood plasma, when the ratio $\dfrac{BHCO_3}{H_2CO_3} = \dfrac{20}{1}$, bicarbonate as a buffer is acting at far from its most efficient point.

The phosphates are more efficient at blood pH. When the ratio $\dfrac{B_2HPO_4}{BH_2PO_4} = 1$, the pH is 6.8, much nearer to the reaction of normal
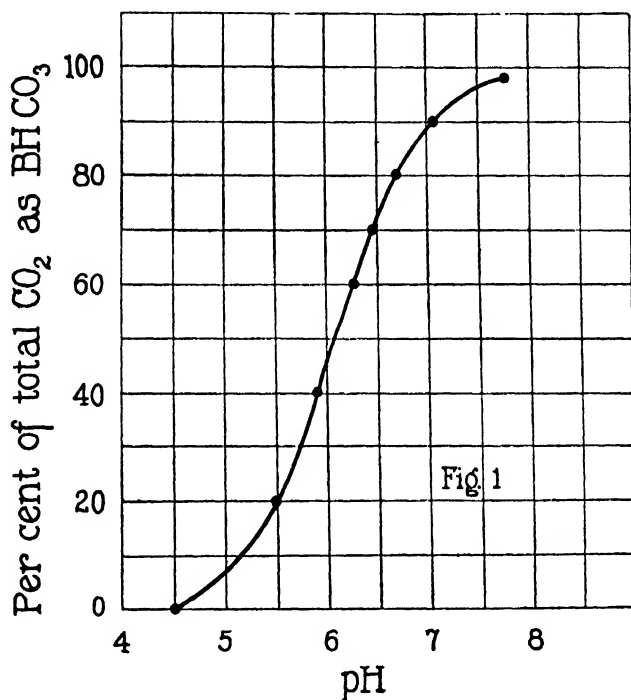
FIG. 1. Action of $NaHCO_3$:H_2CO_3 buffer, showing maximum buffer effect at middle of curve when $NaHCO_3$:H_2CO_3 ratio = 1.

blood than the 6.1 pH of the $BHCO_3$:H_2CO_3 pair when their ratio is unity. The phosphates are present in so small an amount in plasma that they play a quantitatively negligible rôle, but they are more important in the cells. Of all the organic and inorganic acids, other than proteins, that might conceivably be used as buffers by the organisms, L. J. Henderson (1909) estimates that carbonic and phosphoric most nearly approximate maximum efficiency at the blood reaction, despite the fact that the blood pH is not very near the point of maxi-

mum efficiency of either buffer. It is of importance, however, that this point is in both buffers at a lower pH than that normal for blood, so that if the blood pH falls, the phosphate and carbonate buffers oppose the change with an efficiency which increases as the change approaches the danger point. At pH 6.95, which appears to be the most acid point consistent with life in man, the phosphates have very nearly their maximum buffer efficiency.

While the organism has appropriated the best available, although not ideal, buffers that the external laboratories of nature offer, it appears to have manufactured, in the chief blood proteins, buffers of its own which are nearly ideal. For oxyhemoglobin the value of pK_1, and therefore of the pH of maximum buffer efficiency is about 7.16, and for reduced hemoglobin about 7.3, as estimated from data of Haldane, of Joffe and Poulton, and of L. J. Henderson (see p. 553 of this paper).

The Manner in which Buffers Act as Carbon Dioxide Carriers.

We may define a carbon dioxide carrier, in the physiological sense, as a constituent of the blood which increases the amount of CO_2 that may be taken up by arterial blood with a change in reaction equal only to the normal pH difference between arterial and venous blood. Each buffer adds its quota to the amount of CO_2 that can be absorbed within these limits. As stated before, the buffer salts act as reserves of alkali, part of which they give up to neutralize any acid (H_2CO_3 in this case) that is added. The manner in which this action occurs is illustrated by the following example.

Let us assume a solution containing $NaHCO_3$ in 0.03 M concentration, and total phosphate ($Na_2HPO_4 + NaH_2PO_4$) in 0.05 M concentration, the reaction of the solution being pH 7.35, the average normal reaction for blood. We will calculate the changes that occur when the H_2CO_3 is increased sufficiently to lower the pH to 7.25. The molecular ratios $\dfrac{BA}{HA}$, at this reaction are calculated by rearranging the general equation, $pH = pK_1 + \log \dfrac{BA}{HA}$, to the form $\log \dfrac{BA}{HA} = pH - pK_1$. Using for pK_1 in the bicarbonate and phosphate buffers respectively the values 6.10 and 6.80 we have

$$\log \frac{Na_2HPO_4}{NaH_2PO_4} = 7.35 - 6.80 = 0.55$$

$$\frac{Na_2HPO_4}{NaH_2PO_4} = 3.55$$

The total concentration of PO_4 is 0.05 M. Therefore $NaH_2PO_4 = 0.05 - Na_2HPO_4$, whence

$$\frac{Na_2HPO_4}{0.05 - Na_2HPO_4} = 3.55$$

$$\left.\begin{array}{l} Na_2HPO_4 = 0.0390 \text{ M concentration} \\ NaH_2PO_4 = 0.0110 \text{ M concentration} \end{array}\right\} \text{at pH 7.35}$$

In the same way we calculate

$$\left.\begin{array}{l} Na_2HPO_4 = 0.0346 \text{ M concentration} \\ NaH_2PO_4 = 0.0154 \text{ M concentration} \end{array}\right\} \text{at pH 7.25}$$

Since NaH_2PO_4, however, has 1 less Na than Na_2HPO_4, the change from the latter to the former sets free 1 equivalent of Na to combine with H_2CO_3 and form $NaHCO_3$. The concentration of alkali thus freed from the buffer reservoir is calculated as

$$0.0390 \text{ M } Na_2HPO_4 \text{ at pH 7.35}$$
$$0.0346 \text{ M } Na_2HPO_4 \text{ at pH 7.25}$$
$$\text{Difference} = \overline{0.0044} \text{ M Na set free to form } NaHCO_3.$$

At the beginning, with pH 7.35 our $NaHCO_3$ was 0.030 M. We have now by alkali drawn from the phosphates increased it by 0.0044 to 0.0344 M.

We have as additional CO_2 increase added a small amount of free H_2CO_3.

The $\dfrac{NaHCO_3}{H_2CO_3}$ ratio at the start is calculated as follows:

$$\log \frac{NaHCO_3}{H_2CO_3} = pH - 6.1 = 7.35 - 6.10 = 1.25$$

$$\frac{NaHCO_3}{H_2CO_3} = 17.8$$

$$NaHCO_3 = 0.03$$

$$H_2CO_3 = \frac{0.03}{17.8} = 0.00169 \text{ M.}$$

At pH 7.25, the ratio $\dfrac{NaHCO_3}{H_2CO_3}$ is 14.1; hence if the $NaHCO_3$ had remained at 0.03 M, the H_2CO_3 would have been

$$H_2CO_3 = \frac{0.03}{14.1} = 0.00212 \text{ M.}$$

Since the $NaHCO_3$ is increased to 0.0344, however, we have at pH 7.25

$$H_2CO_3 = \frac{0.0344}{14.1} = 0.00244 \text{ M.}$$

The increase from 0.00169 to 0.00212 = 0.00043 M represents the amount of free H_2CO_3 that is added to 0.03 M $NaHCO_3$ solution in changing the pH from 7.35 to 7.25. It is the CO_2 carrying capacity of the bicarbonate alone within this pH range.

Due to the phosphate we have been able to add in addition
$$0.00440 \text{ M } CO_2 \text{ as } NaHCO_3, \text{ and}$$
$$0.00244 - 0.00212 = \underline{0.00032} \text{ M } CO_2 \text{ as } H_2CO_3$$
$$\overline{0.00472} \text{ M } CO_2 = CO_2 \text{ capacity of the phosphate between pH 7.35 and 7.25.}$$

The results are summarized in table 1. The molecular concentrations are transformed into terms of volumes per cent of CO_2 by multiplying by 2240, since 100 cc. of a molecular solution contain 2240 cc. of CO_2 gas.

TABLE 1.

	MOLECULAR CONCENTRATION × 1000		VOLUMES PER CENT CO_2		MILLIMETERS CO_2 TENSION CORRESPONDING TO H_2CO_3
	CO_2 as $NaHCO_3$	CO_2 as H_2CO_3	CO_2 as $NaHCO_3$	CO_2 as H_2CO_3	
CO_2 at pH 7.35.................	30.00	1 69	67.20	3.79	51.9
Increase on changing to pH 7.25					
Due to $NaHCO_3$.............	0.00	0.43	0.00	0.95	13.0
Due to phosphates..........	4.40	0.32	9.86	0.72	9.9
Total increase.................	4.40	0.75	9.86	1.67	22.9
CO_2 at pH 7.25.................	34.40	2.44	77.06	5.46	74.8

We have given this example in such detail because it illustrates exactly the manner in which the buffers of the blood act as CO_2 carriers. Furthermore the bicarbonate concentration in the above is about that of blood plasma, the pH range is normal for blood, and the amount of phosphate present approximates in its CO_2 carrying effect the combined buffers of the blood.

Graphic Measurement of Carbon Dioxide Carrying Capacity of Buffers.

Both the demonstration and measurement of the CO_2 carrying capacity of a buffer solution are facilitated by the use of a graphic method, which we shall later use to demonstrate the CO_2 carriers of the blood, and for the illustration of which the above data will serve.

If we draw a curve, expressing $BHCO_3$ values as ordinates, H_2CO_3 values as abscissae, the curve will be a straight line for all points corresponding to any given $\dfrac{BHCO_3}{H_2CO_3}$ ratio, and the line will rise more or less steeply according as the $\dfrac{BHCO_3}{H_2CO_3}$ ratio is great or small. But a constant $\dfrac{BHCO_3}{H_2CO_3}$ ratio indicates a constant pH. Consequently we are able by a series of straight, slanting lines on a diagram arranged as described to express all possible $\dfrac{BHCO_3}{H_2CO_3}$ ratios and pH values. Diagrams of this nature have been introduced by Haggard and Henderson (1919) for construction of their "CO_2 diagrams" of the blood.

For our purpose, we wish to express as ordinates the total H_2CO_3 + $BHCO_3$ rather than the $BHCO_3$ alone. The rectilinear pH line may still be used, but the calculation of the locus of the line is slightly more complicated. Since it is customary in physiological experiments to express the free CO_2 in terms of CO_2 tension, or the mm. of total atmospheric pressure due to the fraction which consists of CO_2, it is convenient also to use as abscissae units of CO_2 tension. It is permissible to use CO_2 tension in place of H_2CO_3 concentration because, according to the laws of gas solubility, the tension in the gas phase is proportional to the concentration in the solution.

Since at 38° (Bohr, 1905) water in contact with pure CO_2 at 760 mm. tension dissolves 55.5 volume per cent of CO_2, the free CO_2 (or H_2CO_3) of a water solution in equilibrium with CO_2 at 38° and p_{CO_2} mm. of tension may be calculated as:

For water, volumes per cent CO_2 as H_2CO_3 = $55.5 \times \dfrac{p_{CO_2}}{760}$ = $0.073\ p_{CO_2}$.

For blood serum Bohr found that the solubility coefficient was 0.540, slightly less than for water, and for whole blood it was 0.511. Consequently:

For blood serum, volumes per cent CO_2 as H_2CO_3 = $54.0 \times \dfrac{p_{CO_2}}{760}$ = $0.0711\ p_{CO_2}$.

For whole blood, volumes per cent CO_2 as H_2CO_3 = $51.1 \times \dfrac{p_{CO_2}}{760}$ = $0.0672\ p_{CO_2}$.

The total CO_2 content of a water solution under p_{CO_2} mm. of CO_2 tension is therefore calculated as $BHCO_3 + H_2CO_3$ = $BHCO_3 + 0.073\ p_{CO_2}$.

The ratio $\dfrac{BHCO_3}{H_2CO_3} = \dfrac{\text{Total } CO_2 - 0.073\ p_{CO_2}}{0.073\ p_{CO_2}}$.

Designating the ratio $\dfrac{BHCO_3}{H_2CO_3}$ as R we have

$$p_{CO_2} = \frac{\text{Total } CO_2.}{0.073\ (R + 1)}.$$

For locating the pH lines, one calculates R for the desired pH values from the equation log R = pH − 6.10, and then, inserting a convenient value for the total CO_2 in the above equation, calculates the tension, p_{CO_2}, corresponding to the point on the line through which the pH line passes.

If the chart is prepared for whole blood the factor 0.0672 is used in place of 0.073; if for serum or plasma, the factor 0.0711.

The calculation is really simple. For example, if we choose 75 as the total CO_2 line on which to lay off our tension values, the latter are calculated as

$$p_{CO_2} = \frac{75}{0.073\ (R + 1)} = \frac{1027}{R + 1}, \text{ for a water solution, or as}$$

$$p_{CO_2} = \frac{75}{0.0672\ (R + 1)} = \frac{1115}{R + 1} \text{ for whole blood.}$$

Figure 2 is drawn in the manner described. It represents graphically the facts given numerically in Table I.

The level of point A represents the CO_2 content of the solution ($BHCO_3 + H_2CO_3$ = 67.20 + 3.79 = 70.89) at pH 7.35, B represents the content after the tension has been raised sufficiently to change the pH to 7.25. The vertical distance $A'B$ represents the total capacity of the solution to take up CO_2 between the pH limits 7.35 and 7.25. The slight distance from the line AA' to C represents the

CO_2 capacity of the bicarbonate alone, the remainder of the capacity is due to the phosphate.

The slant of the line AC represents the increase in free H_2CO_3 caused by increasing the CO_2 tension, the increase being 0.073 volume per cent for each mm. increase in CO_2 tension.
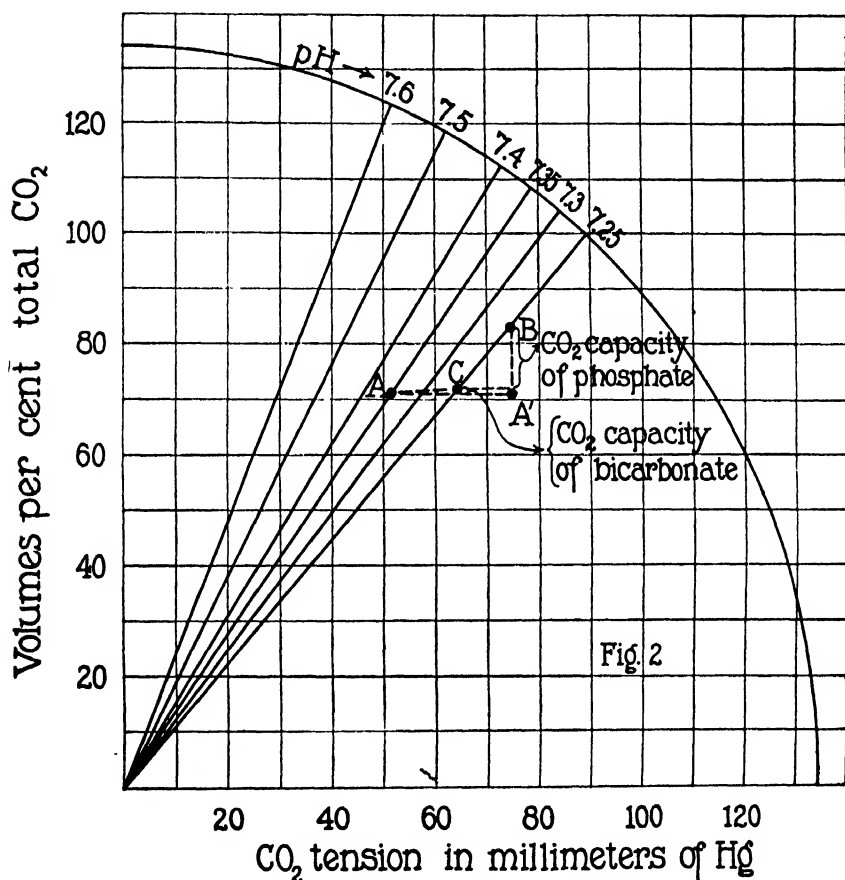
FIG. 2. CO_2 carrying power between pH 7.35 and pH 7.25 (indicated on chart as CO_2 capacity) of a solution containing 0.05 M phosphate and 0.03 M $NaHCO_3$. Vertical distance from line A-A_1 to point B = total CO_2 carrying power. Vertical distance from line A-A_1 to point C = CO_2 carrying power of $NaHCO_3$. Vertical distance from point C to B = CO_2 carrying power of phosphate.

The vertical distance of point C above the line AA' represents the increase in H_2CO_3 sufficient to have brought the bicarbonate solution to pH 7.25, had no buffer other than the bicarbonate been present; it

therefore represents the relatively slight CO_2 carrying capacity of 0.03 M $NaHCO_3$ solution from pH 7.35 to 7.25.

The Carbon Dioxide Carrying Capacity of the Buffers of the Blood.

Experimental evidence that both cells and plasma contain buffers other than bicarbonate and that the cells are several times richer in such buffers than the plasma, was published as long ago as 1868 by Zuntz. He determined the CO_2 absorption curves of whole blood and separated serum, precisely as have Joffe and Poulton (1920), subtracted the physically dissolved CO_2 from the total CO_2, as we have done in this paper in plotting figure 3, and thereby differentiated between free and combined CO_2. His data appear of such interest that we report in table 2 the results of one of his experiments.

The increase in combined CO_2 in whole blood was over three times as great as in serum. Although the dissociation theory was still unborn and the law of mass action unrecognized, Zuntz nevertheless did not fail to understand the significance of his results, and concludes (translation) "From the above it is beyond doubt that the substances which bind carbonic acid in varying proportions belong almost entirely to the cells." Zuntz furthermore was of the opinion that these substances were alkali-protein compounds, and present data indicate that such compounds actually do constitute the most important carbon dioxide carriers in the blood.

The experiments of Zuntz, however, were like some more recent ones, performed over ranges of CO_2 tension such as never occur in the living body, and therefore could not lead to quantitatively accurate conclusions concerning the part which the "CO_2 binding substances" play in the actual physiological regulation of the blood reaction.

Joffe and Poulton (1920) have recently published experments which are of a nature peculiary well adapted to show the effectiveness of the blood buffers as carbon dioxide carriers at reaction ranges within physiological limits. Furthermore their results demonstrate another and most interesting fact, viz., that the richly buffered cells are able, under the influence of changes in reaction, to transfer their buffer effect to the relatively poorly buffered plasma, so that the latter, when in contact with the cells became as efficient a CO_2 carrier as the

former. The cell buffers themselves are chiefly hemoglobin and phosphate. The former cannot diffuse out into the plasma, and the latter does not. There must consequently be some mechanism by which the cell buffers can, so to speak, act at a distance. The mechanism by which this is attained is so important that we shall devote a later section to its discussion.

In figure 3 we have reproduced the CO_2 absorption curves published by Joffe and Poulton, together with pH lines arranged as in figure 2 to make possible a comparison of the effectiveness of the buffers of the three fluids acting over a definite reaction range, pH 7.35 to 7.25.

There are other reliable blood CO_2 absorption curves in the literature from which the CO_2 carrying capacity may be estimated, and the power of the cell buffers to affect the plasma has also been studied.

TABLE 2.

Experiment of Zuntz with Horse Blood and Serum Equilibrated at 0° with Air Containing Varying Percentages of CO_2. Barometer = 758-760.

CO₂ IN AIR	CO₂ IN SERUM		CO₂ IN WHOLE BLOOD	
	Total	Combined	Total	Combined
vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent
22.9	69.3	38.0	95 6	26 3
100.0	180 5	44 3	233 4	47.9
Difference.....	111.2	6.3	137.8	21.6

The peculiar value of the data of Joffe and Poulton for our demonstration, however, lies in the fact that they determined on the same blood: *a*, the amounts of CO_2 absorbed by whole blood under varying CO_2 tensions; *b*, the CO_2 contents of serum samples centrifuged from the blood *after* the latter had been equilibrated at the varying CO_2 tensions (the curve so obtained the authors term the CO_2 absorption curve of "true serum"); *c*, the amounts of CO_2 absorbed by serum centrifugated from the cells of the freshly drawn blood, and then equilibrated, *separately* from the cells, with different tensions of CO_2. This is called the "separated serum."

In constructing figure 3 it has been necessary to draw two pairs of pH lines, óne pair for whole blood, the other for serum, because of the

differences in solubilities of CO_2 in whole blood and serum, respectively, which have been previously discussed in connection with the construction of such diagrams.

A glance at the slopes of the three curves in figure 3 is sufficient to show that the separated serum has only a fraction of the buffer effect, and consequent CO_2 carrying capacity, of the whole blood, while the true serum approximates the whole blood in these respects. The quantitative relationships of the three carbon dioxide carrying capacities are indicated graphically by the vertical distances, indicated by brackets, which show the volumes per cent of CO_2 taken up by whole blood, true serum, and separated serum respectively in passing from pH 7.35 to 7.25. This pH difference is greater than that ordinarily found between venous and arterial blood, but it does not appear to be outside the range of physiological possibilities, and we have preferred it to a shorter range for the present chart, in order that the differences may be large enough to keep the errors of calculation low.

In figure 3 the points SS_1, SS_2, SS_3, etc., have the same relative significance as the points A, C, and B respectively in figure 2. The vertical distance between the horizontal lines on which SS_1 and SS_3 are located indicates the total CO_2 carrying capacity of the separated serum within the pH limits 7.35 and 7.25; the vertical distance between the levels of SS_1 and SS_2 indicates the portion of the CO_2 carrying capacity that is due to the bicarbonate alone. Similar points on the true serum curve are indicated as TS_1, TS_2 and TS_3, respectively, and on the whole blood curve by WB_1, WB_2, and WB_3.

From the data carried from figure 3 to table 3 it is seen that the total CO_2 carrying capacities between pH 7.35 and 7.25 are, for whole blood, 8.0 volumes per cent, for "true serum" 9.4 and for "separated serum" 2.6. When we subtract the amount of CO_2 that the bicarbonate present alone would take up, the values, due to other buffers, are 7.5, 8.8 and 2.0 volumes per cent.

It is evident, first, that within physiological limits as well as under Zuntz's experimental conditions, serum is less than one-third as richly buffered with CO_2 carriers as is the whole blood; and second, that when the same serum is in contact with the cells during a change in reaction, the cells loan it sufficient of their buffer effect to eliminate the difference between them. As a matter of fact, our calculations indicate that in changing from pH 7.35 to 7.25 the true serum took up

even more CO_2 (9.4 volumes per cent) than the whole blood (8.0 volumes per cent).   The difference hardly exceeds the possible error, however, in the difference between two CO_2 determinations, and we can merely say that in the oxygenated whole blood the CO_2 carrying buffer power is about evenly divided between serum and cells, although in actual buffer *content* the serum is less than one-third as rich as the
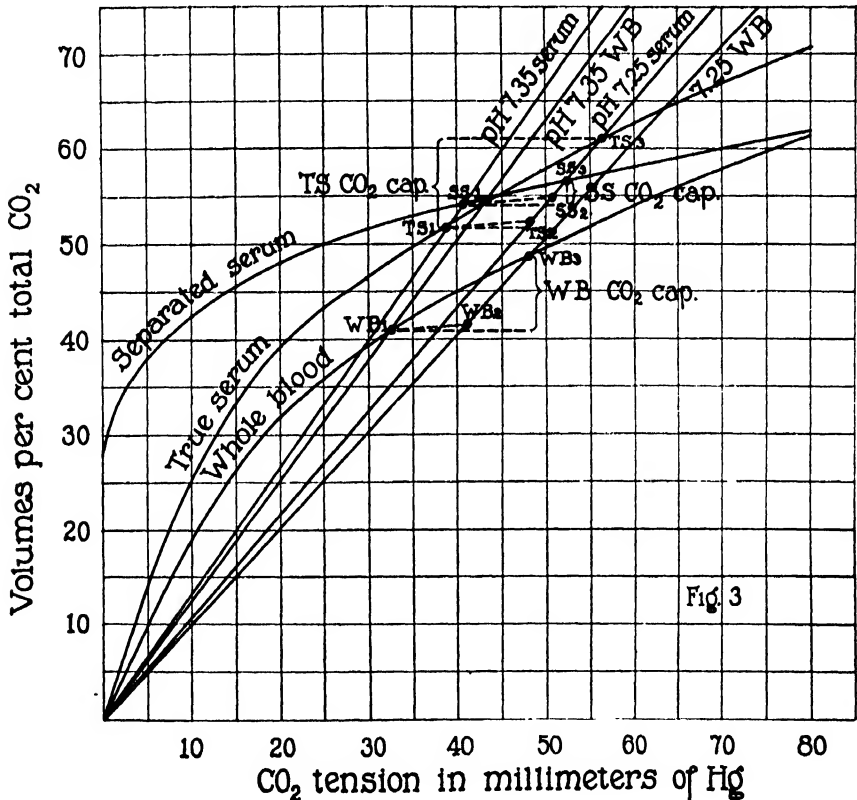
FIG. 3. CO_2 carrying powers (indicated as "CO_2 cap.") between pH 7.35 and pH 7.25 of oxygenated whole blood, of its true serum, and of separated serum. Data of Joffe and Poulton.

whole blood, and therefore presumably less than one-sixth as rich as the separated cells.

Blood has been submitted to fairly complete chemical analyses, and it is practically certain that it contains no substances in considerable amount of which we do not have at least sufficient knowledge to tell

whether or not they can act as buffers, i.e., whether or not they are salts of weak acids or bases. An examination of the constituents reveals among those present in amounts sufficient to have significant effect, only the proteins, the bicarbonate, and the phosphate, which can be expected to act as CO_2 carrying buffers.

The bicarbonate, as indicated by table 3, is responsible for only about $\dfrac{0.5}{8.0}$ or 6 per cent of the total CO_2 carrying power of oxygenated whole blood between pH 7.35 and 7.25.

TABLE 3.

Results Determined from Figure 3.

	WHOLE BLOOD		TRUE SERUM		SEPARATED SERUM	
	CO_2 tension	CO_2 content	CO_2 tension	CO_2 content	CO_2 tension	CO_2 content
	mm.	*vol. per cent*	*mm.*	*vol. per cent*	*mm.*	*vol. per cent*
At 7.35................................	32 4	40.8	38.5	51.6	40.5	54.1
At 7.25................................	48 0	48.8	56.0	61 0	52.0	51 5
Difference..........................	15 6	8.0	17.5	9 4	11.5	2.6
CO_2 carrying capacity due to bicarbonate...		0.5		0.6		0.6
CO_2 carrying capacity due to buffers other than bicarbonate.....................		7.5		8.8		2.0

Of the rôle played by the phosphate we must rely on rough calculation based on unsatisfactory data. So far as we know, determinations of the inorganic phosphate in human blood are not available. Abderhalden (1898), however, published such figures for ox blood, and we use them in want of more direct data. His results indicate 1.30 gram of inorganic P_2O_5 per 1000 grams of corpuscles, equivalent to 0.018 M PO_4''' concentration in the cells. In the plasma the amount is only 0.001 M. To estimate the part which the phosphate may play in the total CO_2 carrying power of the blood, we will assume that the phosphate is divided evenly through cells and plasma, as its buffer effect presumably is, reducing the PO_4''' to 0.009 M, and will compare this amount with that which gives the effect indicated in figure 2. In the latter, it is shown that a phosphate of 0.05 M concentration is capable

of taking up 11 volumes per cent of CO_2 in passing from pH 7.35 to 7.25. A phosphate solution of 0.009 M is only 18 per cent as concentrated, and can be expected to take up about 0.18 as much CO_2 over the same pH range. $0.18 \times 11 = 2.0$ volumes per cent. This would attribute to the phosphates about 25 per cent of the CO_2 carrying capacity of oxygenated blood. We are, however, entirely uncertain as to whether Abderhalden's ox blood figures bear any relation to those of the human blood with which Joffe and Poulton worked, and the above estimation can serve only to indicate the general magnitude of the part that the CO_2 carrying power of phosphates may play in that of the whole blood.

Concerning the part which hemoglobin plays, Campbell and Poulton (1920) have published results which enable us to make an approximate computation. These authors have determined the CO_2 absorption curve of a solution of hemoglobin (laked red cells) which was dialyzed until free from diffusible substances. To the solution thus obtained sodium bicarbonate was added in amount sufficient to make its concentration 0.041 N, which gives a total base concentration to be bound by CO_2 and proteins together of the same order of magnitude as in blood. In figure 4 we have reproduced the curve of the oxyhemoglobin solution so that the CO_2 carrying power between pH 7.35 and 7.25 can be estimated as in figure 2. Since the protein concentration of about 8 per cent approximates that of the plasma, we have used the solubility figures for CO_2 in plasma in drawing the pH lines.

From figure 4 we see that the total CO_2 carrying capacity of the hemoglobin solution between pH 7.35 and 7.25 is about 4 volumes per cent, of which 1.0 volume per cent is due to the bicarbonate, 3 volumes per cent therefore to the hemoglobin. Campbell and Poulton determined that the hemoglobin content of their solution was 52 per cent that of normal blood on the Haldane scale. Hence to compare the buffer effect with that of the hemoglobin in normal blood, we must multiply it by $\dfrac{1}{0.52}$, raising it to 5.8 volumes per cent. In the whole blood (table 3, fig. 3) we found 8.0 volumes per cent of CO_2 carrying power. If all the determinations and calculations were accurate, also the assumption that the blood used by Joffe and Poulton contained 100

per cent of hemoglobin, the above data would indicate that hemoglobin is responsible for $\dfrac{100 \times 5.8}{8.0} = 73$ per cent of the total CO_2 carrying power of the oxygenated blood.

We cannot state too emphatically that the data on which this calculation is based are only semi-quantitative. The exact hemoglobin content of the whole blood used by Joffe and Poulton, which we have used for comparison, is not given; it may have been 90 to 120 per cent
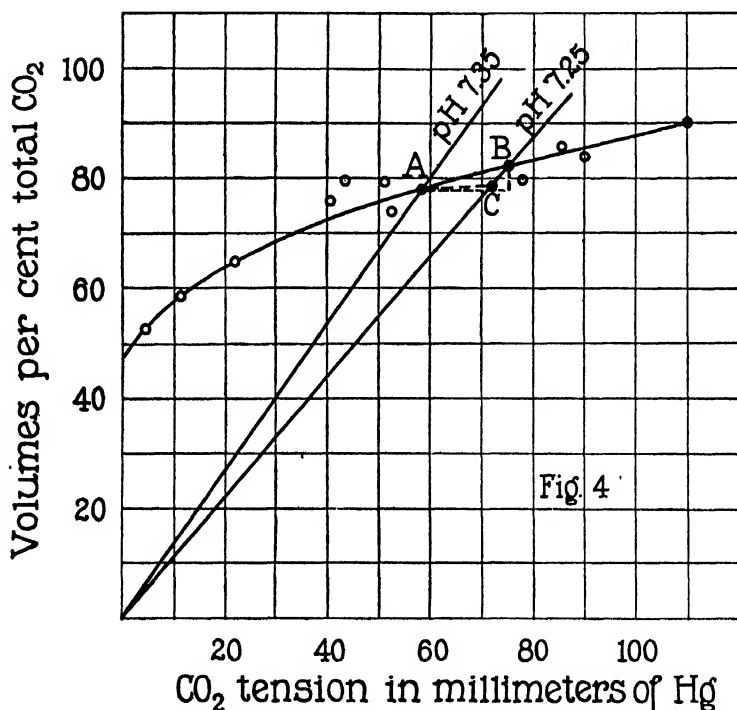
FIG. 4. CO_2 carrying power of a solution of dialyzed hemoglobin. Hemoglobin concentration corresponds to oxygen capacity of 9.6 volumes per cent. Data of Campbell and Poulton.

of Haldane's average normal instead of 100 per cent, as is assumed. Furthermore, the individual CO_2 determinations of Campbell and Poulton on the dialyzed hemoglobin solution are so scattered on both sides of the curve that the accuracy of the latter is uncertain. They are the only data available, however, and uncertain as they are they

indicate the probability that the hemoglobin is chiefly responsible for the buffer effect of the cells, and of the entire blood.

Remembering that the phosphate analysis cited above is from a sample of ox blood, that the hemoglobin in the normal human blood was not determined and is only estimated to be the average normal, and that Campbell and Poulton's dissociation curve for dialyzed hemoglobin is not very accurate, it must be confessed that the data could hardly be less satisfactory for estimating the relative parts that the different buffers play in the total buffer effect of the blood. Taking the data for what they are, however, they indicate the distribution of the blood buffers shown in table 4.

TABLE 4.

Estimated Approximate Distribution of CO_2 Carrying Power among Buffers of Oxygenated Blood.

BUFFER	CO_2 CARRYING POWER OF SEPARATE BLOOD BUFFERS BETWEEN pH 7.35 and 7.25	PROPORTION OF CO_2 CARRYING POWER OF J. J.'S BLOOD
	vol. per cent	*per cent*
Bicarbonate..........................	0.5	6
Plasma protein.......................	1.0	13
Cell phosphate.......................	2.0 ·	25
Oxyhemoglobin.......................	5.8	72
Total by addition....................	9.3	116
Total observed in J. J.'s whole blood (fig. 3)............................	8.0	100

Discussion of the Mechanism by which Buffer Effects are Exchanged between Cells and Plasma.

The experiments of Zuntz (1868) cited above proved that the corpuscles are freely permeable to carbonic acid, but the first evidences that change in CO_2 tension causes a shift of other electrolytes between blood cells and plasma are apparently due to Gürber (1895). Gürber observed that when pure CO_2 is passed through blood the alkali titratable with methyl orange in the serum increases. The

increase was presumably of bicarbonate. Such increase could be due either to diffusion of alkali out of the cells, or of acid into them. By ash analyses Gürber determined that no Na or K had passed into the serum, but that sufficient Cl had left it and entered the corpuscles to account for the increase in serum bicarbonate. The apparent explanation was that when minute amounts of HCl were formed by interaction of H_2CO_3 and NaCl, such HCl at once passed into the cells. Such a phenomenon could be illustrated by the following diagram:

$$H_2CO_3 + NaCl = NaHCO_3 + \left(\overline{HCl}\right) - \left|\begin{array}{c|c}\text{cell} & \text{cell} \\ \hline \text{wall} & \text{interior} \end{array}\right. \longrightarrow$$
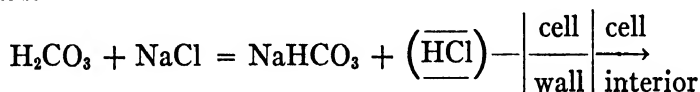
The effect of such a reaction would be to increase the plasma $NaHCO_3$ in response to increase in H_2CO_3, and thereby to cause the increase in the CO_2 carrying power of the plasma over that of separated serum which we have previously discussed in connection with Joffe and Poulton's results.

Gürber's experiments were conducted by passing pure CO_2 through blood, so that the range of CO_2 tension was altogether outside of physiological possibilities. Similar criticism applies to the recent experiments of Fridericia (1920). Van Slyke and Cullen (1917), however, found that the Cl transfer was sufficient to account for 72 per cent of the alkali increase in the plasma that was observed when the H_2CO_3 was raised from 0.0013 to 0.0024 M, both of the latter values being within the physiologically possible range. De Boer (1917) demonstrated that SO_4 anions are transferred in the same way as Cl; but the amount of sulfate in the plasma is so small, 0.002 M, that the proportion of it shifted could hardly have a measurable effect on the total alkali content. Hamburger (1916) has published evidence showing that to some extent cations, Na and K, take part in the shift. The cation shift must be at most, however, slight compared with the anion transfer. It may be stated that the acid-base transfer between cells and plasma is carried out chiefly by the anions Cl' and HCO'$_3$.

Below is a diagram which although from lack of available data is necessarily incomplete, nevertheless appears to represent the chief buffer factors that maintain the constancy of the blood pH, and to

indicate the anion exchange that makes the cell buffers available to the plasma. The equilibria as written are all shifted from left to right by increase in H_2CO_3.

"Na Protein" is used to indicate the total alkali salts of the plasma proteins, "H Protein" the free proteins not combined with base. Similarly "KHbO" and "HHbO" are used to indicate the alkali salt of oxyhemoglobin and the oxyhemoglobin not combined with alkali, respectively. "KHb" and "HHb" are used similarly for reduced hemoglobin. In the plasma the base is indicated as Na, in the cells as K, to indicate the fact that, in human blood at least, these two bases predominate in the two locations, and do not diffuse freely from one to the other. There is, of course, some K in the plasma, and some Na in the cells, which the above diagram fails to show.

PLASMA	CELL WALL	CELL
(1) $H_2CO_3 + NaCl \rightleftharpoons NaHCO_3$ $+ HCl$	$\rightarrow HCl \rightarrow$	(3) $HCl + K_2HPO_4 \rightleftharpoons KH_2PO_4$ $+ KCl$
(2) $H_2CO_3 + Na\ Protein \rightleftharpoons$ $NaHCO_3 + H\ Protein$		(4) $HCl + KHbO \rightleftharpoons HHbO +$ KCl
		(5) $HCl + KHb \rightleftharpoons HHb +$ KCl
	$\rightarrow H_2CO_3 \rightarrow$	(6) $H_2CO_3 + K_2HPO_4 \rightleftharpoons$ $KH_2PO_4 + KHCO_3$
		(7) $H_2CO_3 + KHbO \rightleftharpoons HHbO$ $+ KHCO_3$
		(8) $H_2CO_3 + KHb \rightleftharpoons HHb +$ $KHCO_3$

In regard to the quantitative relationships among the reactions indicated in the diagram, our incomplete knowledge permits the following statements.

Reaction 1, with perhaps lesser reactions which end in other electrolyte transfers to the cells, accounts for two-thirds to three-fourths of the total bicarbonate formed in the plasma of whole blood by a rise in H_2CO_3 such as to cause a pH change within the normal range. This is shown by the fact that an increase in H_2CO_3, sufficient to

change the pH from 7.35 to 7.25, caused in the separated serum of Joffe and Poulton less than one-third as great a rise in $BHCO_3$ as in the true serum, in which the cells were present. The removal of the HCl from plasma by transfer into the cells makes it possible for Reaction 1 to proceed to a measurable degree. When cells are absent it cannot do so, and in the separated plasma only Reaction 2 can occur to a measurable extent.

Reaction 2, it follows, accounts for one-third to one-fourth of the $BHCO_3$ formed in the plasma of whole blood when the H_2CO_3 is increased within physiological limits. In the separated serum, presumably it accounts for practically the whole rise.

In the cells, the sum of the total $BHCO_3$ formed by Reactions 6, 7, and 8 is approximately equal to the $BHCO_3$ formed by Reactions 1 and 2 in the plasma (see for example parallel slopes of "true serum" and whole blood curves in figure 3). If the HCl shift is equivalent to $\frac{2}{3}$ to $\frac{3}{4}$ of the $NaHCO_3$ formed by Reactions 1 and 2, it follows, from the above paragraph, that Reactions 3, 4, and 5 together are equivalent to about $\frac{2}{3}$ to $\frac{3}{4}$ of Reactions 6, 7, and 8 combined. If this proves correct, then, for a given amount of bicarbonate formed in the cells, $\frac{2}{3}$ to $\frac{3}{4}$ as much chloride is taken in from the plasma.

. The CO_2 carrying power of the phosphates in the cells, if PO_4 should approximate the same concentration in human as in ox cells (0.018 M), would be about $\frac{1}{3}$ that of the oxyhemoglobin for a change of pH over the range 7.35–7.25 (see table 4). Reactions 3 and 6 together would then be about equivalent to $\frac{1}{3}$ of the other 4 reactions in the cells. The data on the hemoglobin buffer effect and on the phosphate concentration are so uncertain, however, that the above estimate has only the validity of a rough approximation.

Furthermore, the data used have been obtained entirely with oxygenated hemoglobin, so that only Reactions 4 and 7 with hemoglobin were concerned. By reduction of the oxyhemoglobin, or part of it, so that Reactions 5 and 8 come into play, relationships are essentially altered, since, as previously stated (p. 147) reduced hemoglobin as a buffer is quite different from oxyhemoglobin. The effect of oxygen changes on the buffer action of hemoglobin is of great importance in regulating the blood reaction, and we shall consider it in the following section.

The Part of the Oxygen Exchange in the Carbon Dioxide Carrying Capacity of the Blood.

We have shown that the oxygenated normal blood investigated by Joffe and Poulton could in changing its reaction from pH 7.35 to 7.25 take up 8 or 9 volumes per cent of CO_2. Half as much CO_2 would be about the normal difference between arterial and venous blood in a resting man, and would involve a pH change of only 0.05. The normal arterial-to-venous change was calculated by Parsons (1917) from electrometric measurements, however, to be only 0.02, and Peters and Barr (1921) in three normal men calculated differences of only 0.01 to 0.00. It appears that there is still another factor, besides those which we have studied, which assists in neutralizing the carbonic acid that the blood receives in the tissues.

Christiansen, Douglas and Haldane (1914) were led to a similar conclusion by a comparison of the CO_2 tension changes on the absorption curve of oxygenated blood with the differences estimated between arterial and venous CO_2 tensions in the lungs. The latter averaged 5 to 6 mm., while changes of 15 or 16 mm. were required to make oxygenated blood at 38° *in vitro* take up as much CO_2 as does the venous blood *in vivo*. The added factor in stabilizing the CO_2 tension was demonstrated in a notable paper by these authors to be the change which hemoglobin undergoes as it loses oxygen to the tissues and absorbs it in the lungs while carbon dioxide is passing in the reverse directions. These authors found that normal blood at 38° and any given CO_2 tension within the extreme physiological range (30–70 mm.) absorbed from 5 to 6 volumes per cent more CO_2 if the gas mixture with which the blood was equilibrated was $CO_2 + H_2$, than if the gas mixture was $CO_2 +$ air. The only conceivable effect of the hydrogen as compared with the air was in its effect on the state of oxygenation of the hemoglobin. The conclusion was reached that the change from oxy- to reduced hemoglobin in the tissues enables the blood to take its required load of CO_2 with relatively as small an increase in CO_2 tension as that (5–6 mm.) which these authors calculated from alveolar air analyses.

From the data of Christiansen, Douglas, and Haldane, it is possible to demonstrate, as a matter of fact, that reduction of the blood can

enable it to take up 5.5 volumes per cent of CO_2 and still not only maintain, but increased the alkalinity, because the increased CO_2 is all in the form of bicarbonate. At 40 mm. CO_2 tension, their curves give the total CO_2 contents tabulated below, from which the pH values are calculated as follows:

	TOTAL CO_2 CONTENT AT 40 MM. CO_2 TENSION	CO_2 AS H_2CO_3 = 40 × 0.0672	CO_2 AS $BHCO_3$	pH = $6.10 + \log \frac{BHCO_3}{H_2CO_3}$
	vol. per cent	vol. per cent	vol. per cent	
Oxygenated blood....	51.5	2.69	48.8	7.36
Reduced blood.......	57.0	2.69	54.3	7.40

It is evident that H_2CO_3 must be added to the blood when it is reduced, or it will actually become more alkaline.

If we accept Campbell and Poulton's estimate of the acid dissociation constant of oxyhemoglobin, and use L. J. Henderson's method of estimating the smaller constant for reduced hemoglobin, we may calculate the amount of alkali which the hemoglobin of normal blood would give up, a, in passing from pH 7.35 to pH 7.33; b, in losing its oxygen and changing to reduced hemoglobin. Campbell and Poulton estimated from the curve we have reproduced in figure 4 that when all the hemoglobin in their solution was in the form BHbO, the amount of alkali thus bound was sufficient to combine as bicarbonate with 51.3 cc. of CO_2 per 100 cc. of solution. Since their solution contained only 52 per cent as much hemoglobin as normal blood, the alkali binding power of the hemoglobin in blood may be estimated as equivalent to $\frac{51.3}{0.52} = 99$ cc. of CO_2 per 100 cc. of blood.

Inserting 99 in place of 18 as a constant in Henderson's (1920) calculations, we have $K_O = 6.9 \times 10^{-8}$ and $K_R = 4.9 \times 10^{-8}$. The negative logarithms of these values are $pK_O = 7.16$ and $pK_R = 7.31$. Using these values, we calculate for pH 7.35 as follows, by the method previously illustrated on pages 535–537.

$$\text{Log} \frac{BHbO}{HHbO} = 7.35 - 7.16 = 0.19$$

$$\frac{BHbO}{HHbO} = 1.55$$

$$BHbO = 60.2$$

Similarly at pH 7.33

$$\text{Log} \frac{BHbO}{HHbO} = 7.33 - 7.16 = 0.17, \text{ whence } BHbO = 59.1$$

If we now reduce all of the oxyhemoglobin to reduced hemoglobin, we have with pH still remaining at 7.33

$$\text{Log} \frac{BHb}{HHb} = 7.33 - 7.31 = 0.02, \text{ whence } BHb = 50.6$$

We have therefore yielded

1. By oxyhemoglobin in changing from pH 7.35 to 7.33 sufficient base to combine with $60.2 - 59.1 = 1.1$ cc. of CO_2.

2. By oxyhemoglobin in being altered at pH 7.33 to reduced hemoglobin, sufficient base to combine with $59.1 - 50.6 = 8.5$ cc. of CO_2.

Normally, however, only about one-third of the oxyhemoglobin is reduced in passing from arteries to veins, so we divide the 8.5 cc. by 3, making 2.83 cc. of CO_2 estimated as bound by the alkali set free as a result of the loss of oxygen.

We therefore estimate that the hemoglobin of normal blood, in passing from pH 7.35 to 7.33, and in being one-third reduced from oxy- to reduced hemoglobin, frees sufficient alkali to combine with $1.10 + 2.83 = 3.93$ cc. of CO_2 per 100 cc. of blood. If we assign to the blood buffers other than oxyhemoglobin one-half of its CO_2 carrying capacity over the given pH change, as seems to be approximately correct, they will add 0.55 cc. to the above amount, making 4.48 cc. of CO_2 taken up. To this we must add the CO_2 taken up as H_2CO_3. At pH 7.33 the ratio $\frac{BHCO_3}{H_2CO_3}$ is $\frac{17}{1}$. Consequently in increasing the $BHCO_3$ by 4.48, we increase the H_2CO_3 by $\frac{4.48}{17} = 0.26$ cc., making the total increase $4.48 + 0.26 = 4.74$ cc. of CO_2 per 100 cc. of blood. The

increase in CO_2 tension accompanying the entire change is $\dfrac{0.26}{0.0672}$ =

3.9 mm. The respiratory quotient, $\dfrac{CO_2}{O_2}$ is $\dfrac{4.74}{6.0}$ = 0.79, a normal value.

The maintenance of the normal gas exchange of the organism with a difference of only 0.02 in pH between arterial and venous bloods is therefore explainable on the basis of the available physico-chemical data. The maintenance of an absolutely isohydric exchange, however, would not be thus explainable. It would require that without change in pH the absorption of each cc. of O_2 should cause the loss of at least 0.7 cc. of CO_2, since for each cc. of O_2 consumed the organism forms from 0.7 to 1.0 cc. of CO_2. The data of Christiansen, Douglas, and Haldane (1914) as shown in figure 2 of L. J. Henderson's paper (1920) indicate that at the normal H^+ value of 4×10^{-8} (pH = 7.4) complete loss of O_2 (18 cc.) causes isohydric absorption of only 8 cc. of CO_2, instead of the minimum required volume of $0.7 \times 18 = 12.6$ cc.

L. J. Henderson calculates that, although hemoglobin, like all proteins, is amphoteric and therefore capable of combining with acids, yet under the conditions of CO_2 tension and pH that exist *in vivo*, it cannot possibly combine directly with more than 1 or 2 volumes per cent of CO_2. As CO_2 carriers oxygenated and reduced hemoglobin function not through direct combination of the protein molecule with CO_2, but through acting as buffers, holding reserves of K and Na, which are given off to transform H_2CO_3 into $BHCO_3$ according to the laws of mass action whenever the H_2CO_3 concentration is increased. Further confirmation of this viewpoint is afforded by the results of Campbell and Joffe (1920) who showed that so long as conditions keep the pH within the normal physiological range, addition of oxyhemoglobin to alkali solutions does not increase, but diminishes, the amount of CO_2 combined at a given tension, the oxyhemoglobin competing with the CO_2 for the alkali, which the two substances divide according to their relative strengths as acids. It is only at pH ranges more acid than can exist in the circulation that hemoglobin combines with significant amounts of CO_2. We believe that data in the papers of Henderson, and of Campbell and Poulton, make untenable the view that hemoglobin acts to a significant extent as a CO_2 carrier by combining directly with the CO_2 in the blood.

Haggard and Y. Henderson (1920) have recently thrown some doubt on the validity of applying physiologically the results of Christiansen, Douglas and Haldane. Haggard and Henderson tend to the opinion that the peculiar effect

of oxygenation and reduction on the CO_2 absorption curve of blood may be the result of a property artificially conferred on the blood by the process of defibrination, which was used in all of Christiansen, Douglas, and Haldane's experiments. In support of this view they give data indicating that oxalated blood, which they consider to be less altered from the natural circulating fluid than is defibrinated blood, does not show any oxygen effect on its CO_2 absorption curve. The assumption that oxalated blood is nearer to nature than defibrinated might readily be questioned. The experimental fact that oxalated blood does not show the oxygen effect, however, is one which Peters has attempted to confirm in the writer's laboratory with consistently negative results: defibrinated and oxalated bloods have shown within the limit of experimental error identical oxygen effects on their absorption curves. It does not appear to the writer that any valid reason exists for doubting the physiological significance of the results of Christiansen, Douglas, and Haldane.

The Combined Effect of the Buffers and the Oxygen Exchange in Enabling the Blood to Carry its Carbon Dioxide with a Normal Constancy of Reaction.

It remains to show the combined effect of the factors, which we have discussed in the preceding pages, in enabling the blood to take up the amounts of carbon dioxide which it does in passing from the arteries to the veins with the slight amount of reaction change which is observed.

For this demonstration we use the published CO_2 absorption curves of J. S. H. (Christiansen, Douglas and Haldane, 1914) and of J. J. (Joffe and Poulton, 1920). By means of them we shall estimate the changes which the blood undergoes when, starting completely oxygenated and with a reaction of pH 7.35, it progressively absorbs CO_2 and simultaneously loses volumes of oxygen sufficiently larger to maintain the normal respiratory quotient, 0.8.

In allowing for the effect, discussed in the preceding section, of the change from oxyhemoglobin to reduced hemoglobin on the CO_2 absorption curves, we have used an empirical formula which Peters (Peters and Barr, 1921) has devised. It expresses two assumptions, which hold with a close approximation to constancy over the normal range of CO_2 tension. (1) that the increase in volumes per cent total CO_2 caused by complete reduction of oxygenated blood at constant CO_2 tension is constant; (2) that for partial reductions, the effect on the CO_2 content is proportional to the reduction. In totally reduced blood the oxygen unsaturation is equal to the oxygen capacity, or 18.5 cc. of O_2 per 100 cc. of blood. (The

oxygen unsaturation is the difference between oxygen content and total oxygen capacity, and represents the reduced hemoglobin.) If the increase in CO_2 content caused by total reduction of the hemoglobin is represented by N, the increase in CO_2 caused by an intermediate reduction is estimated as equal to the volume per cent of oxygen unsaturation $\times \frac{N}{18.5}$. From the data of Christiansen, Douglas and Haldane (1914) and of Joffe and Poulton (1920) Peters finds the average value of N for CO_2 tensions between 30 and 70 mm. to be 6.5, and $\frac{N}{18.5} = 0.35$.

If we indicate by D the CO_2 increase due to oxygen loss,

$$D = 0.35 \times \text{volumes per cent of oxygen unsaturation.}$$

A theoretically exact formula could be based on the dissociation constants of the two forms of hemoglobin and the mass law principles expressed in the equations $pH = pK_1 + \log \frac{BHbO}{HHbO}$, and $pH = pK_1 + \log \frac{BHb}{HHb}$, but, considering the uncertainty of the exact value of pK_1 in at least the second of these equations, and the accuracy and simplicity of Peters' empirical formula, the latter seems at present preferable.

The relationship, between the increase in CO_2 content caused by any given increase in CO_2 tension in the oxygenated blood, and the increase in CO_2 content caused when the CO_2 increase is accompanied by an oxygen loss 1.25 times as great, is calculated as follows.

Let $_0\Delta_{CO_2}$ be the increase in CO_2 content of oxygenated blood caused by a given rise in CO tension, without loss of oxygen, and $_R\Delta_{CO_2}$ be the increase in blood CO_2 content caused by the same rise in CO_2 tension, with the simultaneous loss of sufficient oxygen to give a respiratory quotient of 0.8.

O_2 = volumes per cent of oxygen lost, therefore of oxygen unsaturation.

Then $_R\Delta_{CO_2} = {}_0\Delta_{CO_2} + 0.35 \times O_2$.

$$= {}_0\Delta_{CO_2} + 0.35 \times \frac{_R\Delta_{CO_2}}{0.8}$$

Whence $_R\Delta_{CO_2} = 1.78 \, _0\Delta_{CO_2}$

Expressed in words the above equation means that the increase in total CO_2 of the blood, caused by a given rise in CO_2 tension, is further

increased by 78 per cent if the blood loses during the change a volume of oxygen equal to 1.25 times that of the CO_2 it absorbs. In order to plot the actual CO_2 absorption curve of blood as it changes from arterial to venous, we must therefore add to each CO_2 increase measured on the absorption curve for oxygenated blood, 78 per cent more in order to allow for the effect of the normally accompanying oxygen loss.

In each chart (figs. 5 and 6) the heavy black curve AC represents the curve thus calculated, which may be called the true CO_2 absorption curve of blood. The CO_2 absorption curve of oxygenated blood is never touched in the body, save at point A by arterial blood. In the circulation the blood ordinarily travels for about one-third of the length of the true absorption curve from A toward C, and then returns (in the lungs) to its starting point at or near A. Under conditions of stress (e.g., exercise) the venous blood moves further toward C, but apparently never, except in anemic individuals, travels more than about three-fourths of the distance AC.

In figures 5 and 6 we have drawn the pH lines through three points on the true CO_2 absorption curve AC. One is at A, which represents arterial blood. A is arbitrarily located in both bloods, for the purpose of this demonstration, at pH 7.35, the average reaction of normal blood. A second point, B, indicates the conditions attending the absorption of 5 cc. of CO_2, and the loss of 6.3 cc. of oxygen, about the amounts that are ordinarily exchanged during a circulation. The third point, C, indicates the limit of the possible gas exchange, as at this point the blood has lost all of its oxygen (18.5 cc.). In the course of this loss it has absorbed 0.8 as much CO_2, or 14.8 cc.

In table 5 some of the data indicated by measurements on figures 5 and 6 have been put into numerical form. In the "estimated division of CO_2 load among buffers" at the bottom of the table, the estimates are made on the assumption that the relative CO carrying powers of the different buffers of the blood are as represented in table 4 (p. 160). As stated previously, these estimates, especially that concerning the phosphate, are derived from data of only approximate accuracy, but with the possible exception of the phosphate, they may be taken as indicating roughly the relative parts that these factors play in carrying the blood's total load of CO_2.

It is seen from the charts and table 5 that the known factors represented in the curve AC permit both bloods to take up normal amounts of CO_2 with normal reaction changes, the blood of J. S. H. taking up 5 volumes per cent of CO_2 with an increase of 0.03 in pH, that of J. J.
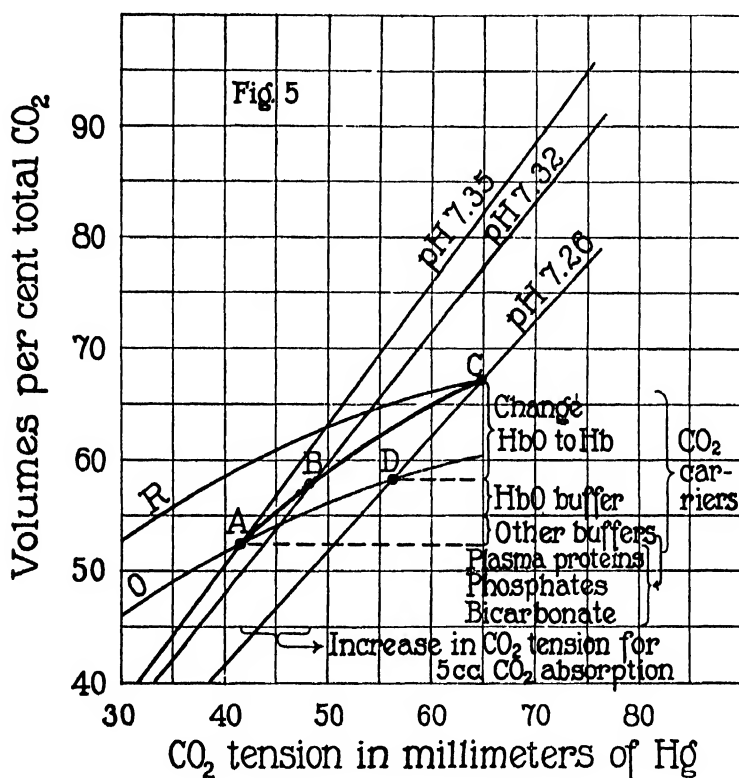
FIG. 5. Relative amounts of CO_2 carried by different agents in blood of J. S. H. The CO_2 absorption curve of oxygenated blood is marked O, that of wholly reduced blood is marked R.

Curve AC represnts estimated absorption curve of blood in the body, when the respiratory quotient is 0.8.   Entire curve AC represents maximum gas exchange, all of oxygen (18.5 cc.) being given off, and 14.8 cc. of CO_2 absorbed, with a pH change from 7.35 to 7.26.

Curve AB represents absorption of 5 cc. of CO_2, with changfe of pH from 7.35 to 732.

AD represents absorption of CO_2 that could occur in change from pH 7.35 to 7.26 if uo hemoglobin were reduced.

The brackets on the right indicate the relative amounts of the added CO_2 neutralized at pH 7.26 by alkali supplied by the different carriers.

with an increase of 0.01. The CO_2 tension increases are also normal, 6.8 and 5.0 mm. It therefore seems probable that we are acquainted with all the CO_2 carriers of importance.
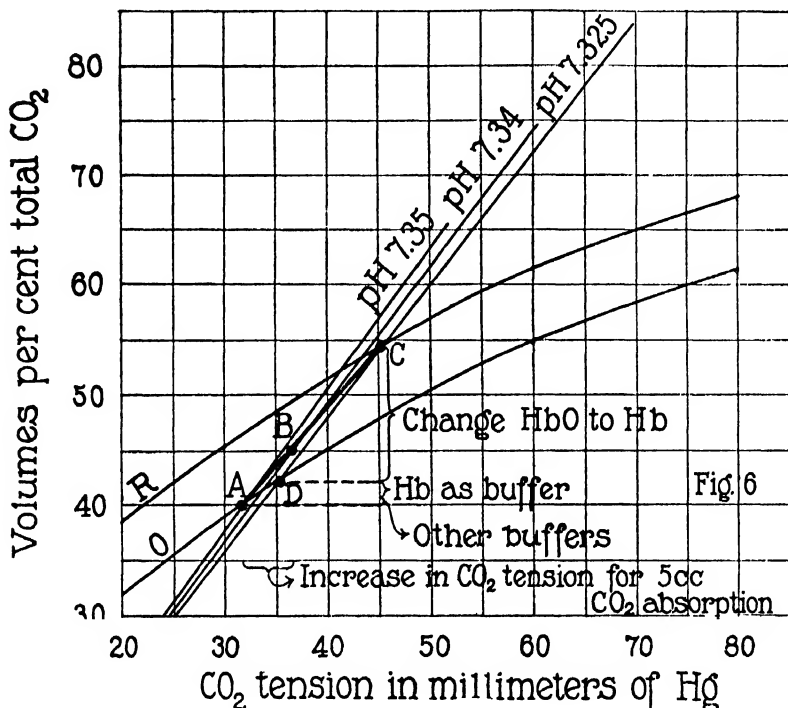
Fig. 6. Relative amounts of CO_2 carried by different agents in the blood of J. J.

The CO_2 absorption curve of oxygenated blood is marked O, that of wholly reduced blood is marked R.

Curve $A\ C$ represents estimated CO_2 absorption curve of blood in the body, when the respiratory quotient is 0.8. Entire curve $A\ C$ represents maximum gas exchange, all of the O_2 (18.5 cc.) being given off, and 0.8 as much, or 14.8 cc. absorbed, with a pH change from 7.35 to 7.325.

Curve $A\ B$ represents absorption of 5 cc. of CO_2, with loss of 6.3 cc. of O_2, increase of 5.0 mm. in CO_2 tension, and change of pH from 7.35 to 7.34.

$A\ D$ represents absorption of CO_2 that could occur in change from pH 7.35 to 7.325 if no hemoglobin were reduced.

If the slope of the CO_2 absorption curves were slightly greater, the line $A\ C$ in the blood would fall upon the line of pH 7.35, and the gas exchange would be accomplished without change in pH. In this case, the HbO→Hb change would furnish all the alkali, the buffers none.

TABLE 5.

	J.B.H.					J.J.				
	Before gas exchange	After +5 cc CO₂ −6.3 cc O₂	After +14.8 cc CO₂ −18.5 cc O₂	Difference after +5 cc CO₂ −6.3 cc O₂	Difference after +14.8 cc CO₂ −18.5 cc O₂	Before gas exchange	After +5 cc CO₂ −6.3 cc O₂	After +14.8 cc CO₂ −18.5 cc O₂	Difference after +5 cc CO₂ −6.3 cc O₂	Difference after +14.8 cc CO₂ −18.5 cc O₂
pH	7.35	7.32	7.26	−0.03	−0.09	7.35	7.39	7.325	−0.01	−0.025
CO_2 tension, *mm*	41.5	48.3	65.0	+6.8	+23.5	31.5	36.5	45.0	+5.0	+13.5
Total CO_2, *vol. per cent*	52.5	57.5	67.3	+5.0	+14.8	40.0	45.0	54.8	+5.0	+14.8
CO_2 increase due to HbO→Hb reaction, *vol. per cent*				3.4	8.8				3.8	12.6
CO_2 increase due to buffers, *vol. per cent*				1.6	6.0				1.2	2.2
Estimated division of CO_2 load among buffers:										
Hemoglobin, *vol. per cent*				1.0	3.7				0.7	1.4
Phosphate, *vol. per cent*				0.4	1.3				0.3	0.5
Plasma proteins, *vol. per cent*				0.2	0.6				0.1	0.2
Bicarbonate, *vol. per cent*				0.08	0.3				0.06	0.1

The point D indicates the rise in CO_2 that, had no hemoglobin reduction occurred, would have resulted within the pH change which actually permitted 14.8 volumes per cent CO_2 increase. The vertical difference in the levels of C and D therefore indicates the volumes per cent of CO_2 carrying capacity that are due to the change from the more acid oxyhemoglobin to the less acid reduced hemoglobin and *vice versa*.

The extent that the buffer factors, other than the change HbO→ Hb, play in enabling the blood to absorb CO_2 within the defined pH limits, is indicated on the charts. It will be noted that, not only is hemoglobin, by virtue of its loss in acidity on reduction, responsible for the greater part of the CO_2 absorption, but also that in carrying the remainder of the CO_2 by buffers, hemoglobin as a buffer plays the major part. The part of hemoglobin in the absorption of the physio- logically maximum amounts of CO_2, indicated by the full distance of the curve AC, is shown in table 6.

It is thus estimated that in the blood of J. S. H. 84 per cent of the total CO_2 carrying power is due to hemoglobin, in that of J. J. 94 per cent.

It is significant that the blood of J. J., in which hemoglobin carried 94 per cent of the CO_2, absorbed its CO_2 quota over a smaller pH range than did the blood of J. S. II. The two facts are necessarily related. The CO_2 carriers, other than the reaction by which oxyhemo- globin changes to reduced hemoglobin and thereby frees alkali to form $BHCO_3$, are simply buffers which give up alkali only when the reaction becomes more acid, and in proportion as it does so. If a blood should absorb CO_2 without any change in pH, which is not theoretically impossible, the sole carrier would necessarily be hemo- globin, because it is unique in its ability to provide alkali without being forced to do so by a rise in hydrogen ion concentration.

The calculations are, especially for J. J.'s blood, subject to certain approximations. We do not know whether the hemoglobin content of J. J.'s blood was the same as that of J. H. S. To the extent that it was different, our AC curve for J. J. is subject to proportional vari- ation. Also we are not certain that 7.35 is the exact pH value nor- mal for J. J.'s blood. Although in a given individual the pH appears to be very constant, in different individuals, according to present data, the pH may vary from 7.25 to 7.45 with 7.35 as the average. If J. J.'s

arterial blood were normal at pH 7.25 instead of 7.35, the relative parts played by his different CO_2 carriers would vary less from those of the blood of J. S. H.   It is regrettable that no figures are available for the purpose of our calculations which include all the desirable data on the same blood.   In the case of the blood of J. S. H., however, it is fairly certain that the range over which we have estimated the changes is normal.   At pH 7.35 J. S. H.'s blood shows an estimated CO_2 tension of 41.5 mm., which is about what J. S. H. considers normal for his thoroughly studied blood.   Also this is the identical blood on

TABLE 6.

	BLOOD OF J. H S.		BLOOD OF J. J.	
	vol. per cent	per cent of total	vol. per cent	per cent of total
Total CO_2 absorbed.....................	14.8	100	14.8	100
By HbO-Hb change......................	8.8	59	12.6	85
By hemoglobin as buffer........	3 7	25	1.4	9
Total absorption due to hemoglobin........	11 5	84	13.0	94

which was based chiefly the calculation of Peters' equation used by us for estimating the effect of reduction of the hemoglobin, so that there appears to be slight danger of error in the estimation of this factor.   Of the two sets of data, therefore, it appears that those on the blood of J. S. H. more certainly represent the exact conditions in the organism than do those of J. J., although it is quite possible that the latter are exact also, and the differences between the two bloods represent genuine individual variation.

CONCLUSIONS.

The carriers of carbon dioxide may be most simply described as substances which hold in combination alkali,. of which they supply to CO_2, as it enters the blood, sufficient to bind nearly all of it as alkali bicarbonate.   When the CO_2 leaves the blood, they recombine with their alkali.   By such reactions, despite changes in CO_2 content and CO_2 tension, the alkalinity of the blood in a given normal individual is maintained constant within marvelously narrow limits.

The chief carbon dioxide carrier of the blood is the hemoglobin. It is almost as completely responsible for the transport of carbon dioxide in the blood as it is for the transport of oxygen. Of the alkali furnished to neutralize the CO_2 that enters the venous blood, from 80 to 95 per cent, sometimes possibly all, comes from the hemoglobin. Of such alkali, the greater part is set free when the relatively strong acid, oxyhemoglobin, is changed by loss of oxygen to the weaker one, reduced hemoglobin. A smaller part of the alkali is furnished by the unchanged oxyhemoglobin, which is itself an efficient buffer at blood reaction. The relatively slight remainder is furnished by the other buffers, viz., the phosphate in the cells, the proteins in the plasma, and the bicarbonate.

The hemoglobin from its location in the cell is able to increase the alkali content of the plasma by withdrawing Cl into the cells from the plasma NaCl, thereby leaving the Na to form $NaHCO_3$. The cells are also freely permeable to the H_2CO_3, so that the latter can enter them to combine with the alkali furnished by the hemoglobin, thereby decreasing the H_2CO_3 of the plasma at the same time that the Cl shift increases its $NaHCO_3$. By means chiefly of the exchange of carbonic and hydrochloric acids with the plasma, the cells are enabled to use their reserves of buffer alkali to maintain constancy of reaction in the plasma, although the buffers themselves (hemoglobin and phosphates) do not leave the cells.

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THE DETERMINATION OF CHLORIDES IN BLOOD PLASMA.

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Van Slyke and Donleavy (1919) have published a method for the determination of chlorides in blood plasma, in which both proteins and Cl are precipitated together by a solution containing picric acid, nitric acid, and standard silver nitrate. The excess silver was titrated in the filtrate by the iodometric method of McLean and Van Slyke (1915). The results in a series of normal human plasmas were identical with those obtained by the method of McLean and Van Slyke, which involved two successive precipitations and filtrations; one of the proteins, the other of the silver chloride. It seemed, therefore, that the Van Slyke-Donleavy method, which involved only one precipitation and filtration, constituted a desirable simplification in the technique for determining plasma chlorides, although it was found still necessary to use the double precipitation for chlorides in whole blood (Austin and Van Slyke, 1920).

We have in the meantime tested the single precipitation method on plasmas from pathological cases by comparing the results with those of the double precipitation method used by Austin and Van Slyke on whole blood, and with those of the total chlorine determination by the Carius method (Table I). In the double precipitation method the preliminary precipitation of the proteins was performed as described by Austin and Van Slyke, except that for 3 cc. of plasma only 10 cc. of picric acid solution are used, instead of the 30 cc. used for 3 cc. of whole blood. The larger amount of picric acid is not needed for plasma, and if employed may result later, when nitric is added, in an undesirable separation of picric acid crystals. The Carius determination was carried out as described by Austin and Van Slyke.

TABLE I.

No.	Appearance of oxalate plasma.	Condition of donor.	Chloride, calculated as NaCl, per liter of plasma.					
			Carius method.		Double pre-cipitation method.		Single pre-cipitation method.	
			Separate determinations.	Average.	Separate determinations.	Average.	Separate determinations.	Average.
			gm.	gm.	gm.	gm.	gm.	gm.
1	Clear.	Normal.	6.02	6 08	6 08	6 08	6.07	6.08
			5.97		6 08		6.08	
			6.16					
			6.15					
2	"	"	6.23	6.27	6.24	6.24	6 32	6.31
			6.31		6.24		6 30	
3	"	"	6.09	6.11			6.07	6.05
			6.13				6.02	
4	"	"	6.06	6.07	5.98	6.03	6.18	6.13
			6.06		6 07		6.08	
			6.08					
5	Deep amber.	Nephritic.	5.29	5 30	5.29	5.30	5.48	5.49
			5.31		5.31		5.49	
6	" "	Cardiac.	5.87	5.92	5 86	5.86	6.05	6.04
			5 97		5.86		6.03	
			5.87					
			5.97					
7	Amber.	Nephritic.	5.87	5.90	5.95	5.95	6.09	6.14
			5.91		5.95		6.19	
			5 92					
8	Creamy opaque.	"			6.02	5.98	6.07	6.08
					5.94		6.08	
9	Clear.	Osteomyelitis.	5.76	5.73	5.72	5.70	5.88	5.87
			5.76		5.68		5.85	
			5.76					
			5.64					
10	"	Arteriosclerosis.	6.06	6.07	5.98	6.01	6.08	6.13
			6.06		6.03		6.18	
			6.08					

CONCLUSIONS.

We have encountered plasma specimens from hospital patients in which the single precipitation gave results indicating a higher chloride content than that obtained when a preliminary removal of the proteins was performed. When such disagreement occurred, the results by the Carius method confirmed those by the double precipitation method of Austin and Van Slyke.

Since the factors which interfere with the single precipitation method in pathological plasmas are not known, it is desirable in all plasmas, both normal and pathological, to remove the proteins by a preliminary precipitation, as in the Austin-Van Slyke method for whole blood, before the chlorides are precipitated with standard silver nitrate.

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STUDIES ON MEASLES.

I. Susceptibility of Monkeys to the Virus of Measles.

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Plate 36.

INTRODUCTION.

Measles occupies a prominent position among the acute infectious diseases that lead to severe and not infrequently fatal secondary infections of the respiratory tract. Efforts to control this serious effect of measles are at present directed almost wholly toward the prevention of the secondary infections by means of isolation and careful treatment of the patient, and while they have met with a certain degree of success, the results obtained are still far from satisfactory. A more hopeful solution of the problem would appear to lie in the prevention of measles itself.

Long experience has shown that the prevention of measles by strict quarantine is largely inadequate because of the contagiousness of the disease during the prodromal period. There would appear to be, however, more than a theoretical possibility that prevention may be accomplished by protective inoculation. This will obviously require isolation of the virus of measles, or at least the development of a method of handling the virus in pure form, whether it is actually identified under the microscope and in the culture tube or not. Much work has already been done in this direction, both by culture study and by attempted transmission of the virus to animals. The former method has not yet yielded positive results. Whether the transmission of the virus to animals has been successfully accomplished has remained an open question because of the conflicting results obtained by different investigators and because of the somewhat indefinite and limited criteria that have been relied upon as evidence

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of a positive transmission. In undertaking a study of measles it has seemed desirable, therefore, to determine at the outset whether more conclusive evidence concerning the susceptibility of animals to the virus of the disease might not be obtained.

This paper presents the results of experiments so far conducted on the transmission of measles from man to monkeys and the passage of the infection from monkey to monkey. The symptomatology and pathology of the reaction produced will be presented in greater detail in a subsequent paper.[1]

LITERATURE.

The more recent experiments upon the transmission of measles to monkeys date from the work of Anderson and Goldberger[2] and are largely based upon Hektoen's[3] earlier report of having successfully transmitted measles from man to man by the subcutaneous injection of ascites broth blood cultures from patients in the early eruptive stage of the disease. The former authors claim to have transmitted the virus of measles to monkeys in a considerable number of instances by the use of whole defibrinated blood, blood serum, and washed corpuscles from cases of measles in the preeruptive or early eruptive stages of the disease. Intraperitoneal, intracerebral, intravenous, and subcutaneous routes of inoculation were employed with apparently equal success. Transmission was also accomplished by subcutaneous injection of nasopharyngeal secretions collected in the prodromal and early eruptive stages, and further transmission from monkey to monkey was carried through six passages. The evidence of a positive transmission consisted in a moderate febrile reaction of inconstant character coming on after an incubation period varying from 6 to 21 days, accompanied in some instances by an erythematous or maculopapular rash of variable character with or without symptoms of rhinitis. No mention is made of Koplik spots, of blood counts, or of histological examination of the skin or mucous membranes. It seems probable that Anderson and Goldberger succeeded in transmitting the virus of measles to monkeys in certain of their experiments, but the evidence which they present is not entirely conclusive.

Nicolle and Conseil[4] using similar methods have also reported the successful transmission of the virus of measles from man to monkey and its further passage

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[1] Blake, F. G., and Trask, J. D., Jr., *J. Exp. Med.*, 1921, xxxiii, 413.

[2] Anderson, J. F., and Goldberger, J., *Pub. Health Rep., U. S. P. H.*, 1911, xxvi, 847, 887; *J. Am. Med. Assn.*, 1911, lvii, 113. Goldberger, J., and Anderson, J. F., *J. Am. Med. Assn.*, 1911, lvii, 476. Anderson, J. F., and Goldberger, J., *J. Am. Med. Assn.*, 1911, lvii, 1612.

[3] Hektoen, L., *J. Infect. Dis.*, 1905, ii, 238.

[4] Nicolle, C., and Conseil, E., *Compt. rend. Acad.*, 1911, cliii, 1522; *Compt. rend. Soc. biol.*, 1920, lxxxiii, 56.

from monkey to monkey. Except for an elevation of temperature after an incubation period of 8 to 11 days no symptoms of measles are mentioned as having been observed in the inoculated monkeys.

Hektoen and Eggers,[5] Tunnicliff,[6] and Lucas and Prizer[7] have studied the leucocyte reaction in monkeys inoculated with blood from measles patients and have shown that a fall in the total leucocyte count takes place. These authors, however, present little definite evidence that the monkeys were infected with the virus of measles.

Jurgelunas[8] inoculated three monkeys with defibrinated blood and five monkeys with secretions of the respiratory tract from cases of measles. He also exposed two monkeys for 5 days to active cases of measles in a hospital ward. None of the animals showed any evidence of infection with the virus of measles.

Sellards and Wentworth[9] and Sellards[10] have recently carried out a series of inoculation experiments in both monkeys and man. Five monkeys were intensively inoculated with large amounts of blood from preeruptive and early eruptive cases of measles. The results were negative. Eight susceptible human volunteers were inoculated with blood from cases of measles in the prodromal or early eruptive stages. None of the men showed any evidence of measles.

Although according to the literature, as Sellards has pointed out, most of the important symptoms of measles have been described in inoculated monkeys, it is striking that no single investigator has obtained all the features in any one animal or even in a series of animals, and that no single symptom has appeared with constancy. The periods of incubation vary widely, the temperature reactions are inconstant, Koplik spots have been noted only by Lucas and Prizer, the rashes described are variable in character and frequently lacking, and there is disagreement in regard to the character of the leucocyte reaction.

EXPERIMENTAL.

In attempting to determine the susceptibility of monkeys to the virus of measles it seemed desirable at the outset to utilize a method that presumably would afford the optimum opportunity for successful transmission; namely, the use of a comparatively large amount of material believed to contain the virus of measles and the inoculation

[5] Hektoen, L., and Eggers, H. E., *J. Am. Med. Assn.*, 1911, lvii, 1833.

[6] Tunnicliff, R., *J. Infect. Dis.*, 1912, xi, 474.

[7] Lucas, W. P., and Prizer, E. L., *J. Med. Research*, 1912–13, xxvi, 181.

[8] Jurgelunas, A., *Centr. Bakt., 1te Abt., Orig.*, 1914, lxxii, 483.

[9] Sellards, A. W., and Wentworth, J. A., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 57.

[10] Sellards, A. W., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 257, 311.

of this material by the natural path of infection. This method might be expected to afford not only the best opportunity for successful transmission, but also a greater probability that the reaction of the animal to the virus would more closely conform to the course of measles as it occurs in man. Clinical observation has indicated beyond reasonable doubt that the virus of measles is abundantly present in the secretions of the respiratory tract during the pre-eruptive and early eruptive stages of the disease and that the respiratory mucous membrane is the natural path of entry of the virus. The method used in the preliminary experiments, therefore, consisted in inoculation of the mucous membranes of the respiratory tract with unfiltered nasopharyngeal secretions of patients in the early stages of measles. The secretions were collected by irrigation of the nasopharynx with 20 to 40 cc. of sterile 0.85 per cent salt solution. Monkeys were inoculated with 5 to 10 cc. of the nasopharyngeal washings by intratracheal injection in order to facilitate retention by the animal of as much of the material as possible. When as much as 5 to 10 cc. was injected a small amount was commonly regurgitated and spread itself over the mucous membranes of the buccal and nasal cavities.

It is appreciated that this method is open to criticism on the ground that many other organisms beside the virus of measles were inevitably introduced into the trachea. It was felt, however, that in most instances these organisms, being largely saprophytic, would be promptly disposed of by the normal respiratory mucous membrane and would not interfere with the ultimate result of the experiment. It furthermore seemed probable that other methods free from the foregoing objection could more satisfactorily be carried out after the susceptibility of the monkey to the supposed virus of measles had been demonstrated and the criteria necessary to establish a positive transmission had been learned. This objection has been met in subsequent experiments by the use of filtered nasopharyngeal washings and of blood shown to be free from ordinary bacteria by culture tests.

The methods of study after inoculation consisted in daily observation of symptoms and inspection of the skin, conjunctivæ, and mucous membranes of the mouth, in the recording of morning and evening temperature (rectal), and daily counts of the white blood corpuscles.

Blood cultures were made at irregular intervals in a variety of media (aerobic and anaerobic) both during the incubation period and during the disease in order to exclude as far as possible the presence of possible intercurrent infections resulting from the introduction of extraneous organisms into the trachea, or occurring independently from other sources. Finally, small sections of skin were excised[11] in nearly all animals during the course of the exanthem in order to determine the histological character of the lesion. All animals were observed for a period of at least 3 weeks after inoculation before being discarded as negative. The evidence for a positive transmission has depended, as in the clinical diagnosis of measles, upon the development of the characteristic symptoms and lesions of the disease, the temperature and leucocyte counts having been recorded merely as additional data and not as evidence of a successful inoculation.

Direct Transmission of the Virus of Measles from Man to Monkeys.

Of ten monkeys inoculated with nasopharyngeal washings from seven patients with measles, eight after an incubation period of 6 to 10 days developed symptoms closely resembling those of measles in man. Of the two monkeys which failed to develop symptoms of measles, one contracted a Pneumococcus Type IV pneumonia with a severe pneumococcus septicemia 24 hours after inoculation and died on the 8th day, while the other failed to show sufficiently definite symptoms to warrant a positive diagnosis and was discarded as negative after 24 days observation. The details of the experiments in which a positive transmission was obtained follow.

In the first two experiments monkeys were inoculated intratracheally with unfiltered nasopharyngeal washings. In the first instance the secretions were collected from the patient about 1 hour after the first appearance of the exanthem, in the second 6 days before the exanthem appeared.

Experiment 1.—Monkey 2 (Text-fig. 1, *a*); *Macacus rhesus.* Mar. 24, 1920. Intratracheal injection of 10 cc. of unfiltered nasopharyngeal washings collected from measles patient, Case 1, about 1 hour after the first appearance of the exanthem. Cultures of the washings showed *Micrococcus catarrhalis, Streptococcus viridans, B. influenzæ, Staphylococcus pyogenes aureus,* and diphtheroid bacilli.

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[11] This was always done under anesthesia.
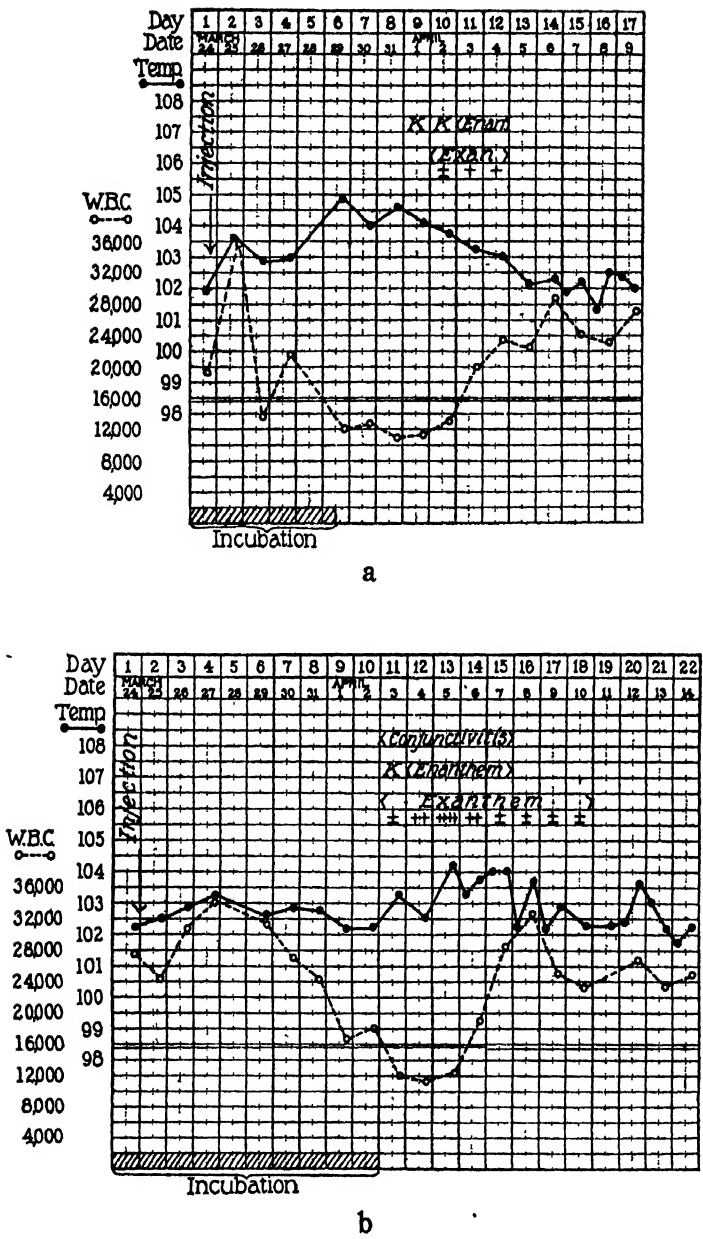
TEXT-FIG. 1, *a* and *b*. Observations on monkeys inoculated with unfiltered nasopharyngeal washings from patients with measles. (*a*) Monkey 2, Case 1. (*b*) Monkey 3, Case 2.

The monkey remained well and active until the 6th day when the temperature rose to 104.7° F. and remained elevated for 5 days. The leucocyte count fell on the 6th day and remained low for 5 days. On the 9th day the animal became less active; there was photophobia, and a small, bright red macule appeared on the mucous membrane of the left lower gum. Blood culture showed no growth. On the 10th day drowsiness was quite marked; two more discrete, erythematous spots were present on the labial mucous membrane and a rash consisting of small, discrete, red maculopapules which faded out on pressure appeared on the forehead, cheeks, and neck. On the 11th day the exanthem was more prominent but had not spread to other parts of the skin. The small hyperemic spots on the labial mucous membrane were more numerous but not coalescent. On the 12th day the enanthem was fading and the exanthem was less marked; by the 13th it had disappeared without definite pigmentation or desquamation and the animal again appeared well and active.

Experiment 2.—Monkey 3 (Text-fig. 1, *b*); *Macacus rhesus.* Mar. 24, 1920. Intratracheal injection of 10 cc. of unfiltered nasopharyngeal washings collected from measles patient, Case 2, 6 days before the appearance of the exanthem. Cultures of the washings showed *Streptococcus viridans, B. influenzæ, Staphylococcus pyogenes albus, Staphylococcus pyogenes aureus,* and diphtheroid bacilli. The animal remained well and active for 10 days. On the 11th day it appeared quiet; the conjunctivæ were markedly congested; a small, bright red macule appeared on the mucous membrane of the upper lip, and in the afternoon a few, small, red, discrete maculopapules appeared on the forehead and cheeks. On the 12th day the conjunctivitis was more marked; there was a moderate, erythematous, granular rash on the mucous membrane of the lips and an abundant, bright red, maculopapular exanthem over the entire face, in places nearly confluent. On the 13th day the animal was weak and drowsy; the exanthem had spread to the flexor surfaces of the arms, forearms, and thighs. On the 14th day the rash had begun to fade. During the 4 following days it gradually disappeared, first from the arms and legs and finally from the face. By the 19th day it had entirely gone, leaving only a yellowish brown pigmentation which lasted for several days and then cleared up. No desquamation was noted. The animal showed a well defined febrile reaction from the 11th to 16th days coincident with the other symptoms. The leucocyte count was low from the 9th to 14th days. On the 3rd day of the exanthem small pieces of skin were excised from the face, left arm, and left thigh. All show the characteristic histology of the measles exanthem, consisting of a proliferative and exudative reaction about the capillaries in the corium. The endothelial cells of the capillary walls are swollen, and numerous endothelial leucocytes are present about the capillaries. A moderate number of these cells are in mitosis, indicating an active multiplication. A few eosinophils, polymorphonuclear leucocytes, and lymphocytes are also present in the pericapillary exudate. There are a few small foci of exudative and degenerative changes in the epidermis. At these points the epithelial cells are swollen and vacuolated, and occasionally show evi-

dence of necrosis. Similar changes are seen in small groups of cells in the hair sheaths and sebaceous glands that lie adjacent to the capillary lesions.

In the next experiment two monkeys were inoculated intratracheally with the pooled nasopharyngeal washings from two patients in the prodromal stage of measles 3 days before the appearance of the exanthem. One monkey received unfiltered washings, the other a portion of the same washings after filtration through a new Berkefeld N filter.

Experiment 3.—Monkey 5 (Text-fig. 2, *a*); *Macacus rhesus.* Apr. 9, 1920. Intratracheal injection of 10 cc. of pooled unfiltered nasopharyngeal washings collected from measles patients, Cases 3 and 4, 3 days before the appearance of the exanthem. Cultures of the washings showed *Staphylococcus pyogenes albus, Micrococcus catarrhalis, Streptococcus viridans,* and diphtheroid bacilli. The monkey remained free from symptoms for 6 days. On the afternoon of the 7th day it appeared quiet and drowsy and shivered at times. On the 8th day two small, hyperemic spots appeared on the mucous membrane of the upper lip. On the 10th day the conjunctivæ were slightly congested and a red, punctate, granular rash was present on the labial mucous membrane. On the 11th day a well defined rash consisting of small, discrete, red maculopapules had appeared on the forehead, cheeks, sides of neck, upper chest, lower abdomen, and inside of thighs. The conjunctivitis remained the same; the enanthem was more marked. On the 12th day the exanthem was more abundant; the individual maculopapules were larger, and fresh spots had appeared over the shoulders. The enanthem was fading. The exanthem rapidly faded on the 2 following days. By the 15th day the animal had completely recovered. No pigmentation or desquamation was noted. There was a well defined leucopenia from the 7th to 15th days. Blood cultures made on the 4th and 7th days showed no growth. Sections of skin removed from the face and thigh on the 2nd day of the exanthem show the characteristic histology of measles.

Monkey 6 (Text-fig. 2, *b*); *Macacus rhesus.* Apr. 9, 1920. Intratracheal injection of 10 cc. of pooled, filtered (Berkefeld N, 20 minutes, 640 mm. vacuum) nasopharyngeal washings collected from measles patients, Cases 3 and 4, 3 days before the appearance of the exanthem. Aerobic and anaerobic cultures of the filtrate showed no growth during 2 weeks incubation except in one of four anaerobic tissue ascites fluid tubes in which a small Gram-negative bacillus, presumably a contaminant, appeared. The monkey remained well and active for 7 days. On the 8th day it showed a moderate conjunctivitis which had increased on the following day. On the 10th day the mucous membranes were congested and showed a moderate, hyperemic, punctate rash on the lips. On the 12th day a few, small, discrete, red maculopapules came out on the forehead, left cheek, and front of the neck. On the 13th day there was a well marked, though sparse,
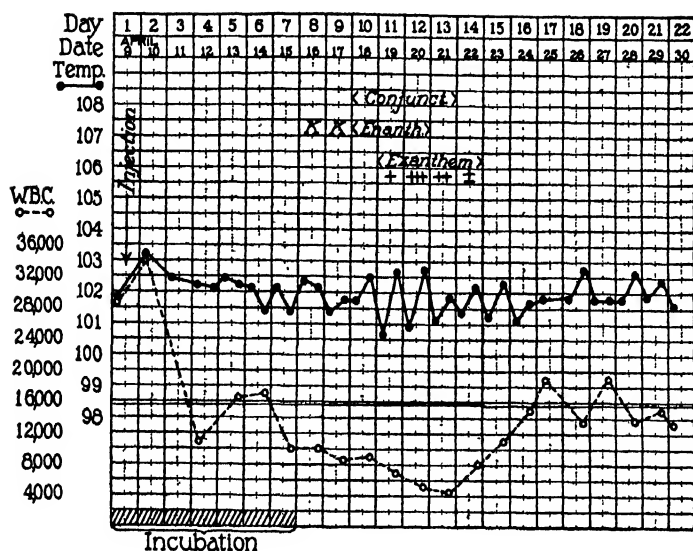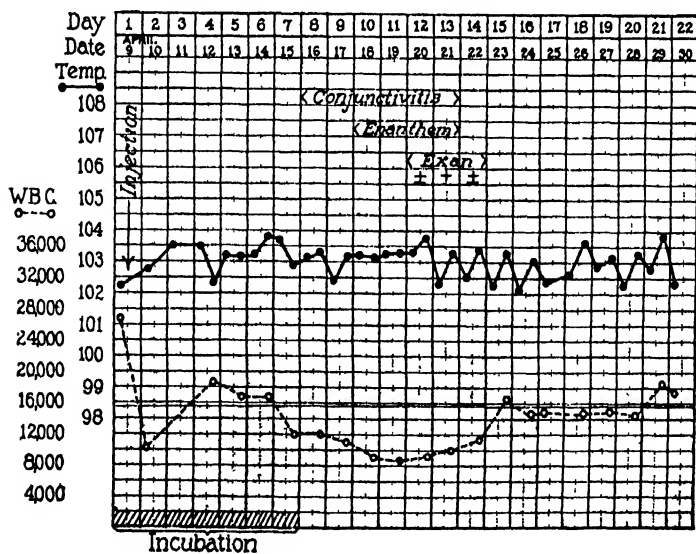
TEXT-FIG. 2, *a* and *b*. Observations on monkeys inoculated with pooled naso-pharyngeal washings from measles patients, Cases 3 and 4. (*a*) Monkey 5, unfil-tered washings. (*b*) Monkey 6, filtered washings.

exanthem on the forehead, cheeks, and neck, and fresh maculopapules had
appeared on the lower abdomen.   On the 14th day the enanthem had disappeared
and the exanthem had begun to fade.   On the 15th day it had disappeared with-
out desquamation; the animal appeared well and active.   There was no febrile
reaction.   The leucocyte count was low from the 7th to 14th days.   Blood cul-
tures on the 2nd, 4th, and 7th days showed no growth.   A piece of skin excised
from the forehead on the 2nd day of the exanthem shows the typical histological
picture of measles.   This animal was subsequently reinoculated on June 8, 1920,
with material containing another strain of the virus of measles.   It failed to react,
the control coming down on the 7th day with characteristic symptoms of measles.

In the following experiment four monkeys were inoculated with
different portions of the nasopharyngeal washings collected from a
patient with measles about 22 hours after the appearance of the
exanthem.   One monkey was inoculated on the mucous membranes
of the nose and throat with unfiltered washings, one intratracheally
with filtered (Berkefeld N) washings, and two intratracheally with
unfiltered washings.   All developed the characteristic symptoms of
measles after the customary incubation period.   The protocols follow.

Experiment 4.—Monkey 8 (Text-fig. 3, *a*); *Macacus rhesus.*   May 12, 1920.
Inoculated on mucous membranes of nose and throat with 5 cc. of unfiltered naso-
pharyngeal washings from measles patient, Case 5.   Cultures of the washings
showed *Staphylococcus pyogenes albus, Streptococcus viridans,* and diphtheroid
bacilli.   The animal remained well for 7 days.   On the 8th day it appeared quiet;
the tongue was coated and its papillæ were prominent; two small, bright red spots
appeared on the mucous membrane of the upper lip.   On the 9th day it was quiet
and limp; the conjunctivæ were injected; the mucous membranes of the cheeks
were congested and a third hyperemic spot was present on the upper lip.   A red,
maculopapular rash appeared on the inner and posterior sides of the thighs and
over the perineum.   On the 10th day the exanthem was more intense and had
spread to the anterior surfaces of the thighs and lower abdomen.   On the 11th
day the exanthem on the thighs had begun to fade.   Fresh maculopapules were
present on the abdomen and on the left cheek.   The conjunctivitis and enanthem
were less marked.   By the 14th day the animal appeared well and the exanthem
had nearly faded, leaving a yellowish brown pigmentation.   There was a well
marked leucopenia coincident with the foregoing symptoms but no definite febrile
reaction.   A section of skin removed from the thigh on the 2nd day of the exan-
them shows the characteristic histological picture of measles.

Monkey 9 (Text-fig. 3, *b*); *Macacus rhesus.*   May 12, 1920.   Intratracheal
injection of 4.5 cc. of filtered (Berkefeld N, 5 minutes, 600 mm. vacuum) naso-
pharyngeal washings from measles patient, Case 5.   Aerobic and anaerobic cul-

TEXT-FIG. 3, *a* and *b*.   Observations on monkeys inoculated with nasopharyngeal washings from measles patient, Case 5.   (*a*) Monkey 8, unfiltered washings. (*b*) Monkey 9, filtered washings.
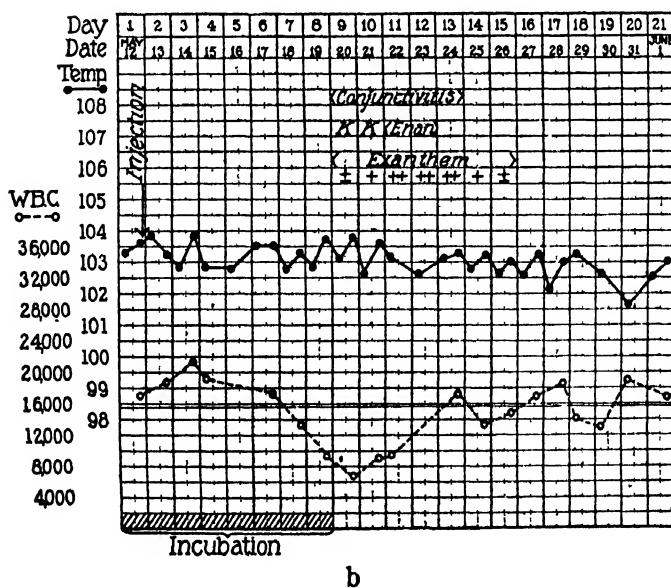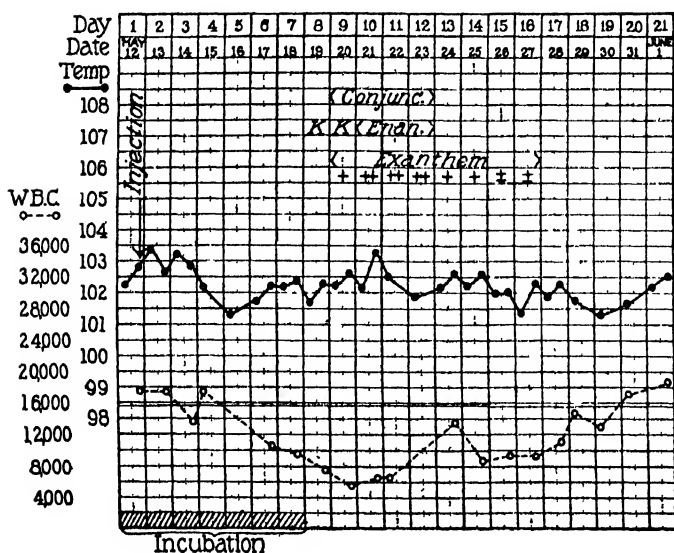
tures of the filtrate showed no growth after 2 weeks incubation. The animal remained well and active for 8 days. On the 9th day it appeared quiet; the conjunctivæ were moderately injected. On the mucous membrane of the upper lip there was a small, red, granular spot and on that of the left cheek there were three slightly elevated, hyperemic spots with bluish white centers presenting the typical appearance of Koplik spots. A few red maculopapules developed on the skin about the left corner of the mouth. On the 10th day the condition was the same, except for an additional spot on the mucous membrane of the lower lip and fresh maculopapules on the left cheek. On the 11th day there was a well developed, red, punctate, granular enanthem on the mucous membrane of the lips; the exanthem had spread to the forehead, cheeks, and nose. On the 13th day the enanthem had cleared up; the conjunctivitis was less marked; the exanthem on the face was fading. A red, maculopapular rash had appeared on the thighs. During the 2 following days the exanthem faded with a fine, branny desquamation, and the animal again became lively. There was no significant febrile reaction, but a well defined leucopenia occurred from the 8th to 11th days. This animal was reinoculated on June 8, 1920, with material containing the same strain of measles virus after it had been passed through three monkeys. It failed to react, while the control after 6 days incubation period developed the characteristic symptoms of measles.

Monkey 10 (Text-fig. 4); *Macacus rhesus*. May 12, 1920. Intratracheal injection of 5 cc. of unfiltered nasopharyngeal washings collected from measles patient, Case 5. The monkey remained well for 7 days. On the 8th day it was listless and drowsy, shivering slightly; a small hyperemic spot was present on the mucous membrane of the lower lip. The tongue was coated and its papillæ were prominent. On the 9th day the conjunctivæ were inflamed; there was a faint erythematous blush over the face and two fresh red macules were present on the mucous membrane of the upper lip. On the 10th day there was a well developed, red, granular enanthem on the labial mucous membrane; a few, small, discrete, red maculopapules appeared on the left cheek and on the upper arms. By the 13th day the exanthem had reached its height. It consisted of numerous, red maculopapules, 2 to 5 mm. in diameter, irregularly scattered over the face, neck, chest, upper arms, abdomen, and thighs (Fig. 1). On the 14th day the conjunctivæ and mucous membranes appeared normal again; the exanthem on the face and neck was yellowish red and showed a fine desquamation. During the 3 following days the exanthem gradually faded with well marked branny desquamation. By the 18th day the animal again appeared well and active. A section of skin removed from the upper arm on the 1st day of the exanthem shows the typical lesion of measles.

Monkey 11 (Text-fig. 5, *a*); *Macacus rhesus*. May 12, 1920. Intratracheal injection of 5 cc. of unfiltered nasopharyngeal washings collected from measles patient, Case 5. The animal remained well until the afternoon of the 6th day when its temperature rose from 103.5° to 104.7° F. and it appeared quiet. On the

7th day it appeared listless and drowsy; the temperature remained elevated. Blood culture showed no growth. On the 8th day there was diarrhea; the conjunctivæ were inflamed and there was increased lacrimation; the mucous membrane of the lips was congested and showed two characteristic hyperemic spots; the temperature rose to 105.8° F. On the 9th day a well defined exanthem consisting of small, discrete, red maculopapules appeared about the eyes, on the nose, right cheek, chin, and about the corners of the mouth. The animal was killed for passage of the virus.
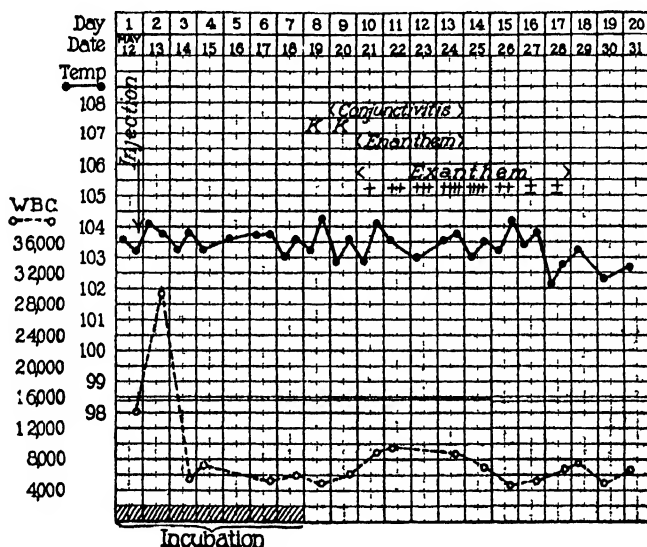
TEXT-FIG. 4. Observations on Monkey 10 inoculated with unfiltered nasopharyngeal washings from measles patient, Case 5.

Autopsy.—Grossly negative. Histological sections of the skin, labial mucous membrane, and tongue show the typical lesions of measles. Cultures of the heart's blood showed no growth.

The foregoing experiments have shown that monkeys injected intratracheally with nasopharyngeal washings from cases of measles in the preeruptive and early eruptive stages of the disease react after an incubation period varying from 6 to 10 days with a constant and characteristic group of symptoms closely resembling those of measles in man. These symptoms in brief are as follows: (1) onset with listlessness and drowsiness after a definite incubation period during which the animal appears well; (2) catarrhal conjunctivitis; (3) a well defined and characteristic enanthem usually confined to the labial

mucous membrane; (4) a definite exanthem consisting of discrete, red maculopapules constant in character though somewhat variable in extent and duration and comparable histologically with the exanthem of measles; (5) leucopenia coincident with the foregoing symptoms; (6) prompt and complete recovery after an illness of 7 to 10 days duration. Variations that have occurred in this group of symptoms are ones of degree in the severity of the reaction rather than in character. In only two respects has the reaction shown any significant variation from the symptoms of measles in man; namely, in the inconstant occurrence of a definite elevation of temperature and in the entire absence of symptoms of rhinitis and bronchitis.

The regularity with which this group of symptoms has developed and the close resemblance of the symptoms to the symptoms of measles are presumptive evidence that the reaction is due to the virus of measles. That the reaction is not due to ordinary organisms of the mouth flora inevitably present in unfiltered nasopharyngeal washings is shown by the occurrence of the same reaction following the inoculation of washings freed from ordinary bacteria by filtration. This, of course, does not exclude the possibility that the reaction might be caused by filterable toxic substances contained in the nasopharyngeal secretions of measles patients rather than by the living virus of measles. This possibility, however, has been excluded by the successful transmission of the infection through a considerable series of monkeys as described below.

Transmission of Measles from Monkey to Monkey by Intratracheal Injection of Tissue Emulsions.

The characteristic reaction which follows the inoculation of monkeys with nasopharyngeal secretions of measles patients has been successfully carried through six passages (Text-fig. 6), by the intratracheal injection of salt solution tissue emulsions prepared from animals killed shortly after the appearance of the exanthem. The principal tissues used were skin and buccal mucous membrane. In some cases bits of spleen, liver, and lung were also employed. In three instances attempted passage failed, presumably due in two to absence of the virus of measles in the material inoculated, in one to the development of a fatal intercurrent infection. The details of the experiment follow.
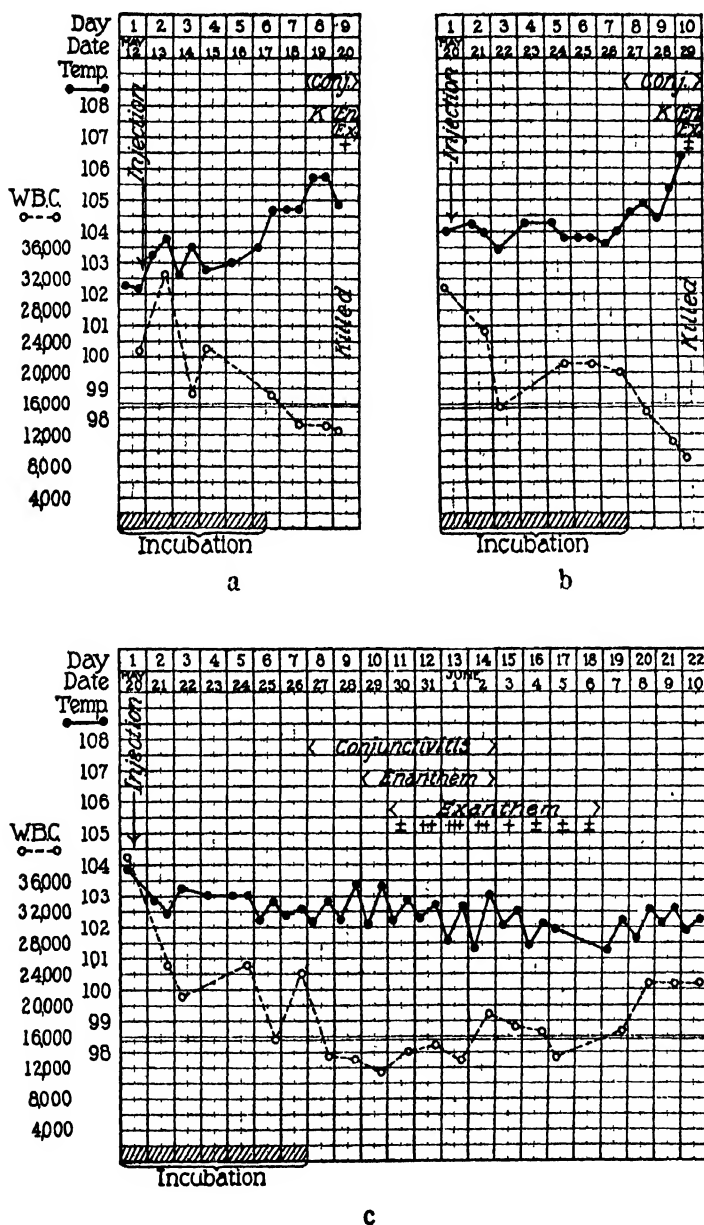
TEXT-FIG. 5, a to c. Transmission of measles virus, strain from Case 5, from monkey to monkey, by means of tissue emulsions. (a) Monkey 11, inoculated with nasopharyngeal washings from measles patient, Case 5. (b) Monkey 13, first passage. (c) Monkey 14, first passage, duplicate of Monkey 13.

Nasopharyngeal washings
from measles patient, Case 5

F.

(8)+   (9)+   (10)+   (11)+
tissue
emulsion

(13)+   (14)+
tissue
emulsion

(16)+   F.
tissue   (17)−
emulsion

(18)−Pn.   (19)+
tissue
emulsion

(22)+   (23)+

blood   blood   blood   blood   tissue
2nd to 4th days 5th to 7th days 8th to 10th days 13th day   emulsion
(24)−   (25)+   (26)+   (27)+   (28)+

tissue
emulsion

(29)+
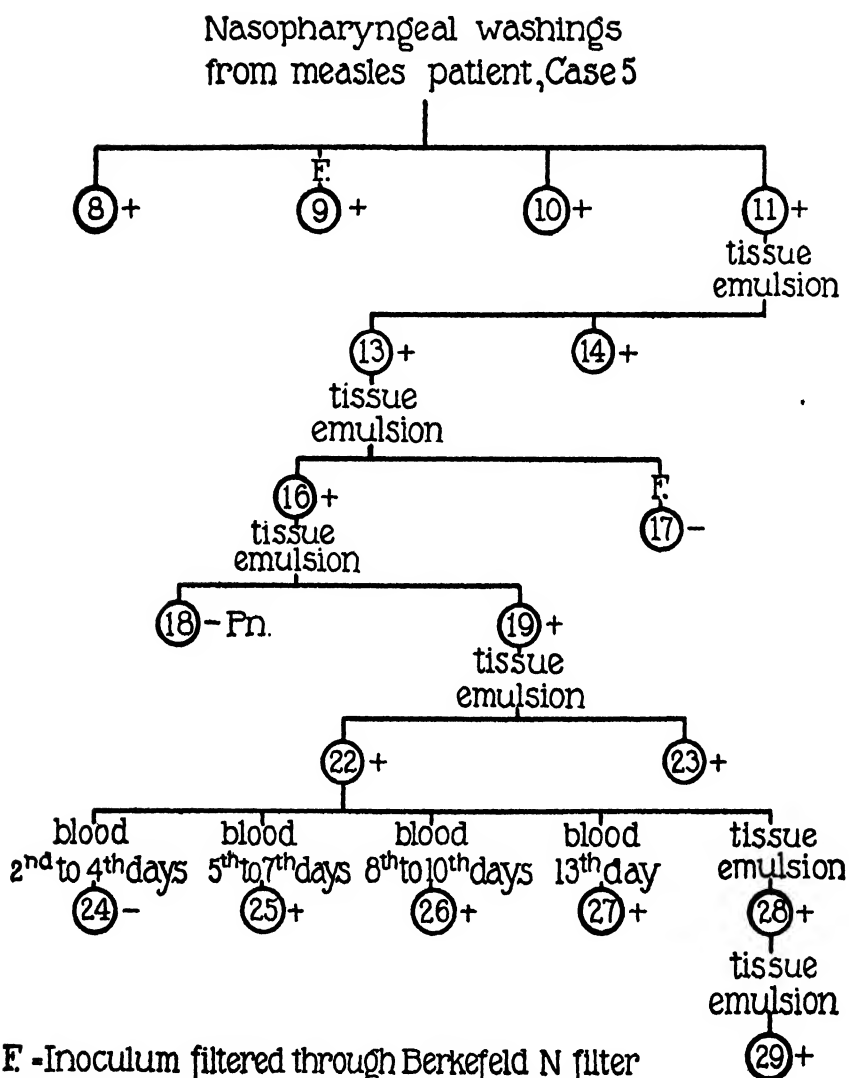
F.−Inoculum filtered through Berkefeld N filter
Pn.−Died with intercurrent pneumonia
+ −Typical measles reaction
− −No reaction

TEXT-FIG. 6. Transmission of measles virus, strain from Case 5, through six animal passages. Monkeys 8 and 23 were inoculated on the nasal and buccal mucous membrane, Monkeys 24 to 27 intravenously, the other monkeys intratracheally. 2nd to 4th days, etc., in Monkeys 24 to 27 indicate the days after inoculation of Monkey 22 on which blood was withdrawn from Monkey 22.

Experiment 5. First Passage.—Monkey 13 (Text-fig. 5, *b*); *Macacus rhesus.* May 20, 1920. Intratracheal injection of 10 cc. of unfiltered tissue emulsion (skin, spleen, liver, and lung) from Monkey 11. Monkey 11 (Text-fig. 5, *a*) was killed on the 1st day of the exanthem, the 9th day after inoculation. The hair was shaved from the face, front of chest, abdomen, and thighs, and the skin thoroughly scrubbed with soap and water. The skin was removed from these areas under aseptic precautions, cut into small bits with sterile scissors and ground with small pieces of spleen, liver, and lung in a sterile mortar with sterile sand and 40 cc. of 0.85 per cent salt solution, the whole procedure requiring about 1 hour. The supernatant fluid of this emulsion was pipetted off and used for inoculation without filtration or centrifugation. Cultures of the fluid on blood agar plates showed a few colonies of *Staphylococcus albus.* Monkey 13 remained well and active for 7 days. On the 8th day the temperature rose from 104° to 104.9° F., the conjunctivæ were inflamed, and the leucocyte count was falling. On the 9th day the animal appeared quiet; a few characteristic spots appeared on the mucous membrane of the lips. On the 10th day the temperature had risen to 106.3° F.; there was a well developed, hyperemic, granular, punctate rash on the labial mucous membrane and a few, red maculopapules had appeared on the face. The animal was killed for further transfer of the infection.

Autopsy.—No gross abnormalities. Histological sections of the skin, tongue, and labial mucous membrane show the typical lesions of measles. Aerobic and anaerobic cultures of the heart's blood, spleen, and lung showed no growth.

Monkey 14 (Text-fig. 5, *c*); *Macacus rhesus.* May 20, 1920. Intratracheal injection of 10 cc. of unfiltered tissue emulsion (skin, spleen, liver, and lung) from Monkey 11 as in the above experiment. The animal remained well for 7 days. On the 8th day it was quiet, showed conjunctivitis and photophobia, and the mucous membranes were congested. No spots were noted. On the 10th day it was drowsy and there was a well developed characteristic enanthem. On the 11th day diarrhea developed; a few small, red maculopapules appeared on the face and thighs. On the 12th day an abundant characteristic exanthem had appeared on the face, neck, chest, shoulders, and thighs. By the 15th day the animal again appeared well; the conjunctivitis and enanthem had cleared up and the exanthem was rapidly fading. Moderate pigmentation persisted for 3 more days and then disappeared. There were a moderate febrile reaction and a well defined leucopenia coincident with the foregoing symptoms. Sections of skin excised from the neck and thigh on the 2nd day of the exanthem show the characteristic histology of measles.

Second Passage.—Monkey 16 (Text-fig. 7, *a*); *Macacus rhesus.* May 29, 1920. Intratracheal injection of 10 cc. of unfiltered tissue emulsion (skin, spleen, and lung) from Monkey 13 prepared in the same manner as in the preceding experiment. Culture of the supernatant fluid showed a few colonies of *Staphylococcus albus.* After the customary incubation period of 7 days the animal developed the characteristic symptoms of measles, the course of which is indicated on the temper-
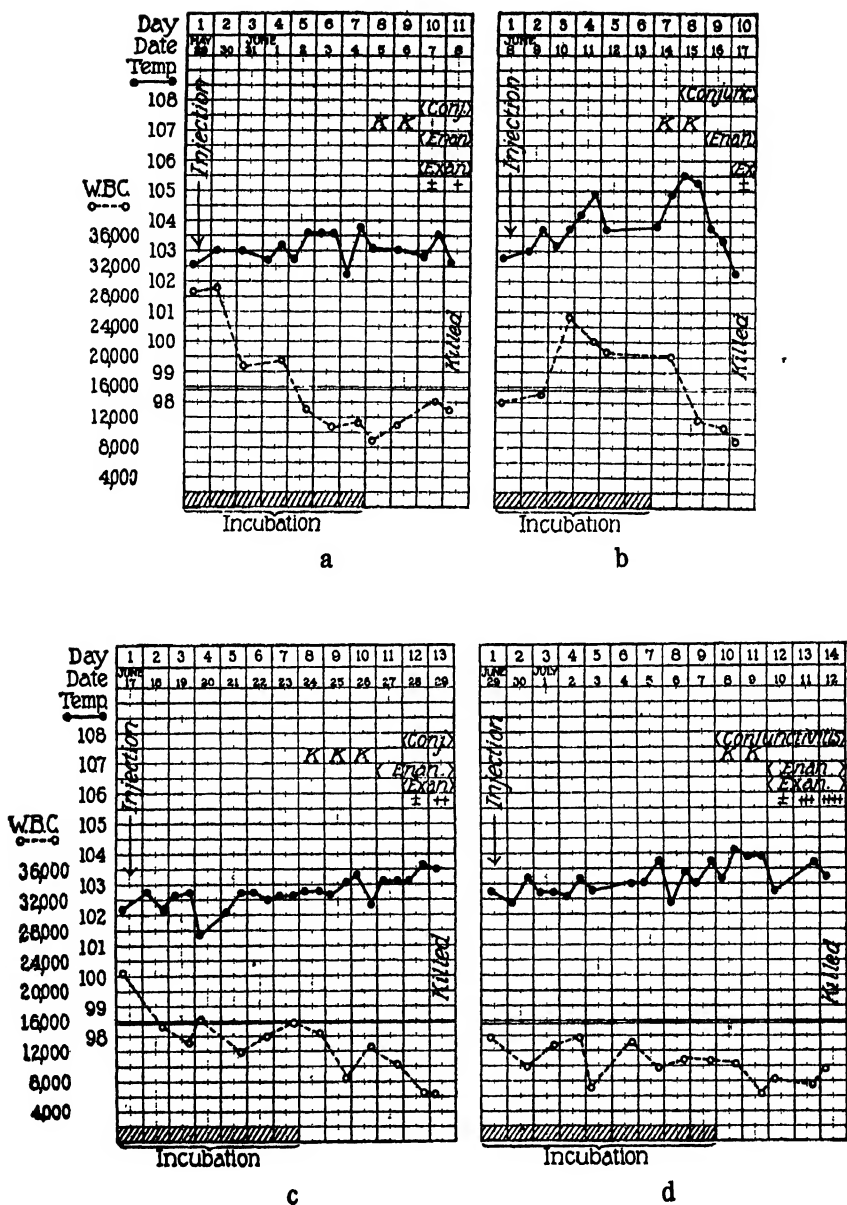
TEXT-FIG. 7, *a* to *d*. Transmission of measles virus, strain from Case 5, from monkey to monkey by means of tissue emulsions. (*a*) Monkey 16, second passage. (*b*) Monkey 19, third passage. (*c*) Monkey 22, fourth passage. (*d*) Monkey 28, fifth passage.

ature chart. The enanthem was very abundant, covering the mucous membranes of the lips and cheeks. The exanthem had appeared on the forehead, nose, chin, and left thigh on the 11th day when the animal was killed for further passage.

Autopsy.—Grossly negative except for a few, small, irregular patches of pneumonia about the hilum of the lungs. Culture showed this to be due to a Gram-positive streptococcus of the *viridans* group. Histological sections of the skin and labial mucous membrane show the characteristic lesions of measles.

Monkey 17; *Macacus rhesus.* May 29, 1920. 20 cc. of the tissue emulsion from Monkey 13 were centrifuged at low speed for 10 minutes, the supernatant fluid was diluted with 40 cc. of salt solution and then passed through a Berkefeld N filter (15 minutes, 600 mm. vacuum). Filtration was slow and after about 20 cc. had passed through it was stopped. 10 cc. of the filtrate were inoculated intratracheally. The monkey remained free from definite symptoms and was discarded as negative after 21 days observation.

Third Passage.—Monkey 18; *Macacus rhesus.* June 8, 1920. Intratracheal injection of 10 cc. of unfiltered tissue emulsion (skin, mucous membrane of mouth, spleen, and lung) from Monkey 16. Cultures of the emulsion showed a few colonies of *Streptococcus viridans* and *Staphylococcus albus.* This animal promptly developed a severe pneumonia with *Streptococcus viridans* septicemia followed by multiple arthritis and died on the 21st day without exhibiting any definite evidence of measles.

Monkey 19 (Text-fig. 7, *b*); *Macacus rhesus.* June 8, 1920. Intratracheal injection of 10 cc. of unfiltered tissue emulsion from Monkey 16 as described above. This animal also developed pneumonia but the disease was mild in character. Blood culture on the 5th day showed no growth and the monkey appeared to have recovered. On the 7th day a few, discrete, hyperemic spots appeared on the mucous membrane of the lips. On the 8th day the animal was quiet; the conjunctivæ were injected; fresh spots had appeared on the mucous membranes of the cheeks. On the 9th day there was an abundant, bright red, granular rash on the mucous membranes of the lips, gums, and cheeks. On the 10th day a few, red maculopapules appeared about the lips, on the chin, and behind the ears. The animal was killed for further passage.   .

Autopsy.—Grossly negative except for a small patch of pneumonic consolidation in the right lower lobe. Cultures of this area showed *Streptococcus viridans.* Histological sections of the tongue and labial mucous membrane show the typical lesions of measles.

Fourth Passage.—Monkey 22 (Text-fig. 7, *c*); *Macacus rhesus.* June 17, 1920. Intratracheal injection of 6 cc. of unfiltered tissue emulsion (skin and mucous membranes of lips and cheeks) from Monkey 19. Cultures of the tissue emulsion showed a few colonies of *Staphylococcus albus*, *Streptococcus viridans*, and diphtheroid bacilli. The animal remained well for 7 days. On the 8th day it was quiet; several small, discrete, hyperemic macules with whitish centers appeared on the mucous membrane of the lips. On the 11th day a red, granular rash ap-

peared on the gums.   On the 12th day there were moderate conjunctivitis and an abundant enanthem on the mucous membranes of the lips, gums, and cheeks which appeared deeply congested, granular, and covered with minute whitish points. A few, small, red, maculopapules developed about the lips.   On the 13th day the exanthem had spread to the arms, abdomen, thighs, and lower legs.   The animal was killed for further passage.   Blood cultures made daily from the 2nd to 10th days showed no growth.

Autopsy.—Grossly negative.   Sections of skin, mucous membrane of the lips, and tongue show the typical histology of measles.   Cultures of the heart's blood showed no growth.

Monkey 23; *Macacus rhesus.*   June 17, 1920.   Mucous membranes of nose and throat inoculated with 10 cc. of unfiltered tissue emulsion (skin and buccal mucous membrane) from Monkey 19.   The animal remained well for 6 days.   From the 7th to 14th days it appeared quiet and drowsy.   It showed a mild conjunctivitis from the 8th to 14th days with photophobia on the 10th, 11th, and 12th days. A few, discrete, hyperemic macules were present on the mucous membrane of the lips from the 7th to 10th days.   No diffuse enanthem and no exanthem appeared at any time.   The leucocyte count was low from the 6th to 16th days, varying between 9,460 and 15,740 cells per c.mm., while previous and subsequent counts ranged from 17,460 to 22,800 per c.mm.   Although it seemed probable that this animal suffered from a mild infection, the clinical evidence was insufficient to warrant a definite conclusion.   Histological sections of the labial mucous membrane, however, show the typical lesion of measles.

Fifth Passage.—Monkey 28 (Text-fig. 7, *d*); *Macacus rhesus.*   June 29, 1920. Intratracheal injection of 6 cc. of unfiltered tissue emulsion (skin and mucous membranes of lips and cheeks) from Monkey 22.   Cultures of the emulsion showed a few colonies of *Staphylococcus albus*, diphtheroid bacilli, and a Gram-negative diplococcus.   The animal remained well for 9 days.   On the 10th day it was listless; the conjunctivæ were injected; a small hyperemic spot appeared on the mucous membrane of the upper lip.   On the 11th day several discrete spots of similar character appeared on the mucous membrane of the lower lip.   On the 12th day there were a well developed characteristic enanthem and a faint eruption of discrete, red maculopapules over the chin, neck, and inner surfaces of the thighs. On the 13th day there was an abundant, well developed, typical exanthem over the face, neck, shoulders, chest, thighs, and back of lower legs.   On the 14th day the condition was the same and the animal was killed for further passage.

Autopsy.—Grossly negative. . Cultures of heart's blood showed no growth. Sections of skin, tongue, and labial mucous membrane show the typical histological picture of measles.

Sixth Passage.—Monkey 29 (Text-fig. 8); *Macacus rhesus.*   July 12, 1920. Intratracheal injection of 8 cc. of unfiltered tissue emulsion (skin, mucous membranes of lips and cheeks, and piece of tongue) from Monkey 28.   Cultures of emulsion showed a few colonies of *Staphylococcus pyogenes aureus*, a non-hemo-

lytic streptococcus, and a Gram-negative bacillus.   The animal remained well for 7 days.   On the 8th day the temperature rose from 102.9° to 103.8° F.; four discrete hyperemic spots were present on the mucous membrane of the upper lip.   On the 10th day diarrhea developed; the conjunctivæ were moderately congested. On the 11th day the conjunctivitis was more marked; a red, granular, punctate rash was present on the mucous membranes.   On the 14th day the enanthem was very marked, involving the mucous membranes of lips and cheeks.   There were marked conjunctivitis and photophobia.   On the 15th day a few, discrete, red maculopapules appeared about the lips.   On the 16th day the exanthem was more marked, but had not spread.   The enanthem was fading.   The temperature had returned to normal.   Killed for further passage.
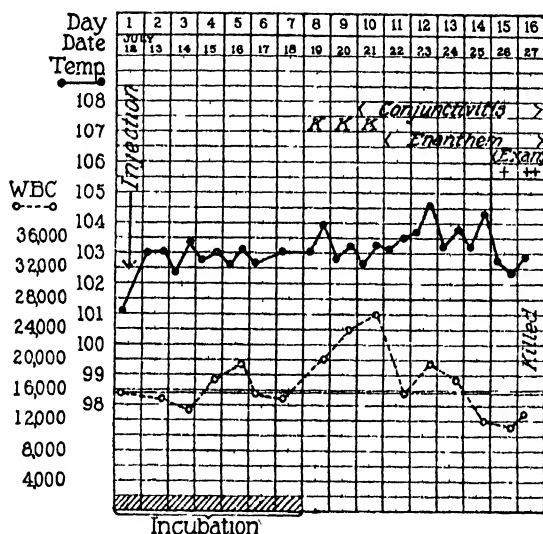
TEXT-FIG. 8.   Observations on Monkey 29, sixth passage of measles virus, strain from Case 5.

Autopsy.—Grossly negative.   Cultures of heart's blood showed no growth. Histological sections of skin and labial mucous membrane show the typical lesions of measles.

Seventh Passage.—Monkey 30; *Macacus rhesus.*   July 27, 1920.   Intratracheal injection of 8 cc. of unfiltered tissue emulsion (skin and mucous membrane of lips) from Monkey 29.   This monkey showed little evidence of infection.   It was killed on the 14th day, however, in an unsuccessful attempt at further passage. It seems probable that Monkey 29 was killed too late for successful transmission.

The foregoing experiments have shown that the characteristic group of symptoms which occurs in monkeys following inoculation with nasopharyngeal washings from patients with measles is readily

induced in monkeys by intratracheal injection of salt solution emulsions of the skin and buccal mucous membranes of monkeys killed early in the course of the reaction.   The transmission of this group of symptoms through six animal passages makes it highly probable that the reaction is due to the living virus of measles.   In the use of unfiltered emulsions of the skin and buccal mucous membranes, as in the use of unfiltered nasopharyngeal washings of patients, other organisms than the virus of measles were unavoidably introduced into the trachea.   Although there is no evidence that these bacteria were in any way associated with the characteristic measles reaction, it is obviously desirable to use a method of transmission that will exclude the presence of extraneous organisms as a possible source of error in the interpretation of the results obtained.   This was attempted in the case of Monkey 17 by filtration of the tissue emulsion, but without success, since under the conditions of the experiment the virus apparently either failed to pass the filter or was so diluted that it failed to give rise to infection.   The desired result has been accomplished, however, by the use of blood.

Transmission of Measles from Monkey to Monkey by Intravenous Injection of Citrated Whole Blood.

The clinical course and pathological lesions of measles clearly suggest that the virus is present in the blood stream for at least an appreciable length of time.   The experiments of Hektoen, Anderson and Goldberger, and Nicolle and Conseil, if the conclusions of the authors are accepted, support this view.   To obtain further knowledge on this subject as well as to eliminate the undesirable features of the method of transmission employed in the passage experiments described above, the following experiment was carried out.

Experiment 6.—Monkey 22 (Text-fig. 7, *c*), which had been inoculated intratracheally on June 17, 1920, with 6 cc. of unfiltered tissue emulsion from Monkey 19, was bled daily from the 2nd to 10th days after inoculation and again on the 13th day.   The blood, which was received into 2 to 3 cc. of sterile 1 per cent sodium citrate solution, after being cultured, was immediately injected intravenously into other monkeys as follows (Text-fig. 6): The 2nd, 3rd, and 4th day bleedings, in amounts of 10, 7.5, and 12 cc. respectively, were injected into Monkey 24.   This
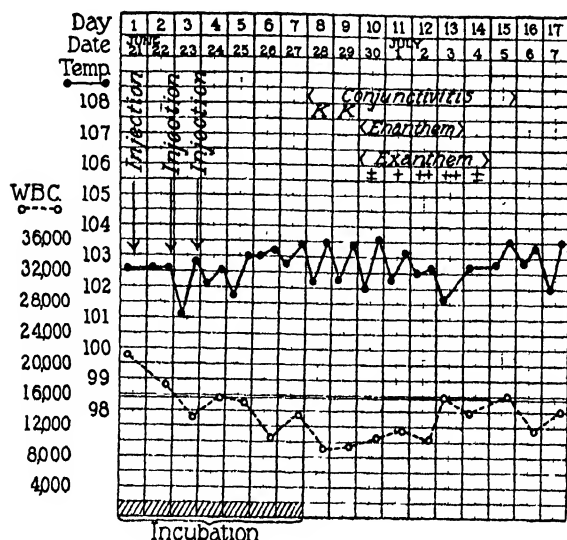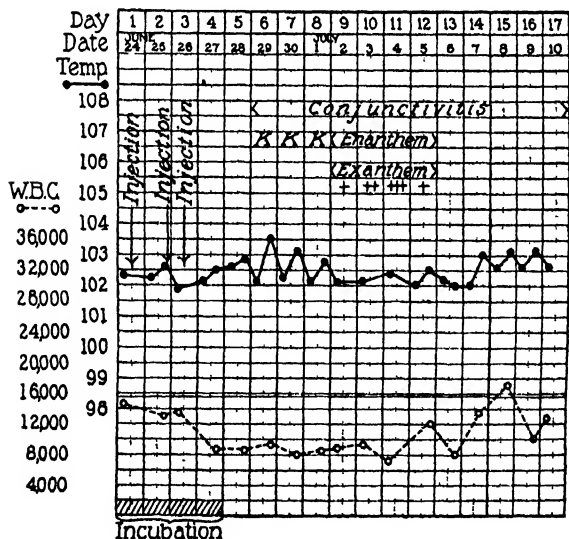
TEXT-FIG. 9, *a* and *b*. Transmission of measles virus, strain from Case 5, from Monkey 22 by means of whole citrated blood. (*a*) Monkey 25, injected with 5th, 6th, and 7th day bleedings from Monkey 22. (*b*) Monkey 26, injected with 8th, 9th, and 10th day bleedings from Monkey 22.

animal remained free from any symptoms of measles and was discarded as negative after 25 days observation. The 5th, 6th, and 7th day bleedings, in amounts of 9, 8, and 9 cc. respectively, were injected into Monkey 25. This monkey remained well until the 8th day after the first inoculation of blood when it developed the characteristic measles reaction, the course of which is shown in Text-fig. 9, *a*. The 8th, 9th, and 10th day bleedings, in amounts of 12.5, 6, and 13 cc. respectively, were injected into Monkey 26, which fell ill after an incubation period of 4 days with the typical measles symptoms (Text-fig. 9, *b*). The 13th day bleeding, 20 cc., was injected into Monkey 27. This animal likewise showed a typical
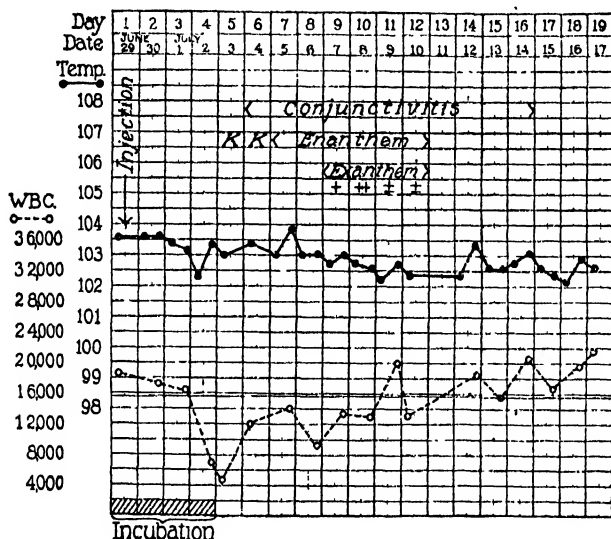
TEXT-FIG. 10. Observations on Monkey 27, injected with the 13th day bleeding from Monkey 22. Measles virus, strain from Case 5.

reaction (Text-fig. 10), after an incubation period of 4 days. Cultures of all bleedings from Monkey 22 were made in agar plates. None showed growth. Sections of skin excised from Monkeys 25, 26, and 27, during the period of the exanthem, all show the characteristic histological picture of measles.

The result of the foregoing experiment is important in that it satisfactorily eliminates the objection that can be raised to the preceding passage experiments on the ground that other organisms beside the virus of measles were present in the tissue emulsions employed. It furthermore shows that in monkeys inoculated intratracheally with measles material the virus subsequently enters the blood stream and can be transmitted readily from monkey to monkey

by intravenous injection of whole citrated blood. This observation is of considerable value because it provides a simple method of transmitting the virus from animal to animal in a pure state and affords an opportunity for extensive culture study of a material known to contain the virus of measles and presumably free from other organisms. There are other suggestive points in the experiment which lend support to previous conceptions of the course of measles in man. The failure of Monkey 24 to react suggests that an appreciable period intervenes between the time of infection of the mucous membranes of the respiratory tract and the entrance of the virus into the blood stream. The positive transmission to Monkey 25 shows that the virus had entered the blood of Monkey 22 by at least the 7th day after inoculation, a time which corresponded with the end of the incubation period of the disease and preceded the onset of symptoms by 24 hours. Whether the virus was present in the blood of Monkey 22 on the 5th and 6th days it is impossible to say. It is of interest in this connection that the incubation period in Monkey 25 was 7 days, while in Monkeys 26 and 27 it was but 4 days. This suggests the possibility that the virus was not present in the 5th and 6th day bleedings from Monkey 22. The difference in incubation period, however, may be explained on the assumption that the virus was much more abundant in the blood of Monkey 22 from the 8th to 13th days, which coincided with the active stage of the disease, than from the 5th to 7th days, which coincided with the latter half of the incubation period. The positive results in Monkeys 26 and 27 show that the virus was present in the blood from the onset of the disease until at least the 2nd day of the exanthem. How much longer it may persist in the blood has not as yet been determined. The shortening of the incubation period in Monkeys 26 and 27 from the customary 7 days to 4 days was presumably dependent upon the introduction of the virus directly into the blood stream, since by this method of inoculation the period of several days which probably elapses between the inoculation of the virus on the mucous membranes and its invasion of the blood is eliminated. Although our observations on the infectivity of the blood have so far been made only on the experimental disease in monkeys, it seems probable that similar conditions will be found to obtain in measles in man.

SUMMARY.

By the intratracheal injection into monkeys of unfiltered nasopharyngeal washings from cases of measles in the preeruptive and early eruptive stages of the disease a relatively constant group of symptoms was induced which closely resemble those of measles in man. Of seven monkeys inoculated intratracheally with unfiltered nasopharyngeal washings from seven cases of measles, five developed the symptoms. The same group of symptoms was induced in one monkey by inoculation of the mucous membrane of the nose and mouth with unfiltered nasopharyngeal washings from a case of measles. In these experiments a variety of organisms, largely saprophytic inhabitants of the nasopharynx and mouth, were present in the material inoculated. There is sufficient evidence, however, that these organisms were in no way responsible for the reaction, since the same group of symptoms was induced in two monkeys by the intratracheal injection of nasopharyngeal washings from three cases of measles after the washings had been freed from ordinary organisms of the mouth flora by filtration through Berkefeld N filters.

The characteristic group of symptoms which follows the inoculation of monkeys with the nasopharyngeal washings from patients with measles has been successfully carried through six passages by intratracheal injection of saline emulsions of the skin and buccal mucous membranes of monkeys killed from 2 to 6 days after the onset of the reaction. From the fourth passage monkey the reaction was also successfully induced in three monkeys by means of citrated whole blood injected intravenously. This experiment showed the blood to be capable of inciting the reaction from at least the 7th to 13th days after intratracheal inoculation of the donor monkey, but incapable of inducing it from the 2nd to 4th days. Cultures of the blood showed no growth.

The group of symptoms induced has been constant and definite in character. After an incubation period of 6 to 10 days the animal becomes listless and drowsy, the conjunctivæ become injected, and small, discrete, hyperemic macules appear on the labial mucous membrane. These spots increase in number and may eventually coalesce in the course of 2 to 4 days to form a diffuse, red, granular

rash. This rash is usually limited to the labial mucous membrane but may extend to the inside of the cheeks. The individual macules may or may not show the minute bluish white center characteristic of Koplik spots. From one to several days after onset an eruption of small, discrete, red maculopapules appears on the skin, usually coming out first on the face. The rash progressively increases in the number and size of the individual lesions and may in the course of 2 to 3 days extend to the skin of the neck, shoulders, upper arms, chest, abdomen, and thighs. It is constant in character but varies considerably in extent in different animals. By the time the exanthem is fully developed, the rash on the mucous membranes has begun to fade and soon disappears. The exanthem in turn progressively fades, sometimes with a branny desquamation, sometimes without. There may be moderate pigmentation. By the 6th to the 10th day after onset all symptoms have disappeared and the animal again appears well. Coincident with this group of symptoms there is a constant and definite reduction in the total leucocyte count, frequently constituting a true leucopenia. Other symptoms of irregular occurrence are photophobia, diarrhea, and fever. Symptoms of rhinitis and bronchitis have not been noted. Histological sections of the lesions of the skin show an exudative and proliferative lesion about the capillaries of the corium in which endothelial leucocytes are the predominating cells. Mitotic cells are not infrequently present in these areas. The endothelial leucocytes may be seen migrating toward and occasionally invading the epithelial layers of the hair follicles, sebaceous glands, and epidermis. In places the epithelial cells appear edematous and vacuolated, and there is evidence of minute vesicle formation. The lesions of the labial mucous membrane are similar in character. Minute vesicle formation in the epithelium is more frequent and the vesicles occasionally assume a more pustular appearance. Similar lesions are found in histological sections of the tongue. Cultures of the blood made both during the incubation period and during the course of the reaction in a variety of media, aerobic and anaerobic, have consistently shown no growth.

The close similarity of the symptoms and pathological lesions of the reaction to the symptoms and pathological lesions of measles, the successful transmission of the reaction from monkey to monkey,

and the elimination of ordinary bacteria as a possible source of error in the interpretation of the results, warrant the belief that the reaction is caused by the inciting organism of measles.

CONCLUSION.

Monkeys (*Macacus rhesus*) are susceptible to inoculation with the virus of measles.

EXPLANATION OF PLATE 36.

Fig. 1. Exanthem in Monkey 10, inoculated with the nasopharyngeal washings from a patient with measles. The drawing illustrates the character and distribution of the lesions on the 3rd day after the first appearance of the exanthem.

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STUDIES ON MEASLES.

II. SYMPTOMATOLOGY AND PATHOLOGY IN MONKEYS EXPERIMENTALLY INFECTED.

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PLATES 37 TO 41.

It has already been shown[1] that a group of symptoms closely resembling those of measles is readily induced in monkeys by inoculation with the nasopharyngeal secretions of patients in the pre-eruptive or early eruptive stages of measles, and that the virus inducing this reaction is readily transmitted from monkey to monkey by inoculation with blood or tissue emulsions from monkeys experimentally infected. In this paper the symptoms and course of the reaction and the histology of the lesions of the skin, labial mucous membrane, and tongue will be described.

Symptomatology.

Incubation Period.—In monkeys inoculated on the mucous membranes of the respiratory tract the incubation period is remarkably constant, being 7 days in the majority of animals. It may vary, however, from 6 to 10 days. Thus in sixteen monkeys inoculated on the respiratory mucous membrane, the incubation period was 6 days in four, 7 days in nine, 8 days in one, 9 days in one, and 10 days in one. During this period the animal appears well and exhibits no recognizable symptoms. The incubation period after intravenous inoculation is apparently somewhat shorter (4 days), but enough animals have not been inoculated by this method to warrant a definite statement.

[1] Blake, F. G., and Trask, J. D., Jr., *J. Exp. Med.*, 1921, xxxiii, 385.

Onset and Course.—The onset is indicated by listlessness, loss of appetite, drowsiness, and diminution in the total leucocyte count. There may be a sharp rise in temperature, but this is not constant. The conjunctivæ become injected, and small, discrete, hyperemic macules appear on the labial mucous membrane. During the following 2 or 3 days these increase in number and may eventually coalesce to form a diffuse, hyperemic enanthem on the mucous membrane of the lips and cheeks. From 1 to 5 days after onset a red, maculopapular rash appears on the skin, usually coming out first on the face. The rash increases and may eventually spread to the skin of the neck, arms, chest, abdomen, and thighs, reaching its height in 2 or 3 days. By this time the enanthem is fading. The exanthem in turn progressively fades, and by 6 to 10 days after onset all symptoms have disappeared.

Conjunctivitis.—An acute catarrhal conjunctivitis is exhibited by nearly all animals during the period of the reaction. This conspicuously involves the inner canthus and tarsal conjunctiva, the bulbar conjunctiva remaining little if at all affected. It varies from a moderate congestion of the vessels to a diffuse hyperemia. In the more severe cases there are increased mucoid secretion, increased lacrimation, and definite photophobia. Moderate edema of the lids may be present. Purulent inflammation does not occur.

Enanthem.—Coincident with or shortly following the onset of the reaction a definite and characteristic enanthem appears. This begins with the development of one or more discrete, red spots on the labial mucous membrane. These spots usually occur only on the labial mucosa, but may occasionally be present on the inside of the cheeks and on the gums. They do not occur on other parts of the buccal mucosa. They present the appearance of discrete, bright red, slightly elevated, lusterless macules, 1 to 2 mm. in diameter. Occasionally they show a minute, bluish white center. They occur either singly or in small groups of two or three. Their number progressively increases during the first 2 or 3 days of the reaction. By the end of this time there may be ten to fifteen in all. From this point the enanthem pursues one of two courses. In the milder infections the spots now begin to fade and in 1 to 2 days have disappeared. · In more severe infections, however, the spots coalesce to form a more

diffuse, red, slightly granular rash covering large areas of the labial mucous membrane with intervening areas of pale pink mucosa which appear to be normal. In still severer infections the entire labial mucous membrane and the inside of the cheeks finally become covered with a diffuse, bright red, granular rash studded with numerous minute, white, slightly depressed specks. The enanthem now begins to fade and rapidly disappears in the course of 2 to 3 days.

Exanthem.—From 1 to 5 days, usually on the 3rd or 4th day, after onset a characteristic exanthem appears. It generally comes out first on the face, especially about the eyes, the corners of the mouth, and on the cheeks, but may also appear approximately at the same time on other parts of the body, particularly on the inner surface of the thighs. It begins with a few, discrete, red maculopapules occurring either singly or in small groups. The individual maculopapules are 2 to 3 mm. in diameter, fade out gradually at their margins into the surrounding normal skin, and are frequently located about a hair follicle. They disappear on pressure and are never hemorrhagic. There is no diffuse erythema of the intervening skin, which appears normal. The further progress of the rash shows the characteristic evolution of the measles exanthem, though the rash itself rarely becomes as widespread or as thick as it commonly does in man. Generally in the course of 2 or 3 days the rash comes out progressively on the skin of the neck, chest, lower abdomen, and the inner surfaces of the upper arms and thighs. While it is usually limited to these less hairy areas of the skin, it may occasionally be sparsely present on the scalp, shoulders, back, forearms, and posterior surface of the legs. During this period the individual maculopapules tend to increase in size, but they never become sufficiently large or thickly aggregated to produce a confluent rash. Having reached its height the rash begins to fade and disappears in the course of 2 to 5 days. This sometimes takes place without noticeable desquamation, but there frequently is a fine, branny desquamation of the skin over the central portion of the maculopapules. Occasionally a slight, yellowish brown pigmentation may persist for 2 or 3 days after the complete fading out of the hyperemia. While the foregoing description presents a composite picture of the exanthem, the extent of the rash and the number of maculopapules vary considerably in individual animals.

Thus it may vary from a comparatively sparse exanthem limited to the face or face and inner surfaces of the thighs, to a widespread, moderately thick exanthem involving the skin of the face, neck, trunk, and extremities. The character of the individual maculopapules, however, remains constant and typical.

Fever.—There is the widest possible variation in febrile reaction. The temperature may rise abruptly at onset, even to as high as 105–106°F. On the other hand, the disease may begin without fever and remain so throughout. When fever occurs it may persist only during the prodromal stage before the appearance of the exanthem or may continue throughout the course of the disease. It may be distinctly remittent in type or fairly well sustained. In an occasional animal the preliminary rise at onset is followed by a return to normal and a distinct secondary rise. In these cases the temperature curve closely resembles that of measles in man.

Leucopenia.—A definite diminution in the total leucocyte count consistently occurs coincident with the reaction. This usually begins 1 or 2 days before the onset of symptoms and persists for several days until the height of the exanthem is reached. At this time the leucocytes begin to increase and gradually return in the course of 3 days to a week to their former level. The degree of leucopenia varies somewhat in different animals, but it is nearly always well defined and may be very marked, many animals showing counts as low as 4,000 to 9,000 cells per c.mm. over a period of several consecutive days. No significant change in the leucocyte count which may be attributed to the virus of measles appears to occur during the earlier part of the incubation period. Animals inoculated with unfiltered nasopharyngeal washings usually show a leucocytosis for 24 to 48 hours after inoculation, but this is undoubtedly due to the effect of other organisms, since it does not occur in animals inoculated with filtered washings in which other organisms than the virus of measles are presumably not present. It has not as yet been determined whether the diminution in leucocytes is due to a greater reduction in one type of leucocyte as compared with other types.

Other Symptoms.—The general symptoms of malaise, such as loss of appetite and activity, are usually moderate in degree, but may occasionally be quite marked. They begin with the onset of the

reaction and persist until the exanthem begins to fade. A very definite drowsiness is often present during the first 2 or 3 days of the disease. Another symptom of not infrequent occurrence is a moderate diarrhea during the earlier part of the reaction. The entire absence of symptoms of rhinitis and bronchitis should be noted.

Microscopic Pathology of Skin, Labial Mucous Membrane, and Tongue.

The tissues available for study consisted of small pieces of skin excised,[2] during life from twelve monkeys during the period of the exanthem and pieces of skin, labial mucous membrane, and tongue obtained at autopsy from seven monkeys killed for passage of the virus. The tissues were fixed in Zenker's fluid and stained with alum-hematoxylin and eosin. A composite picture of the lesions will be given.

Skin.—Lesions are present both in the corium and in the epidermis. The reaction in the corium (Figs. 1 to 6) is definite and typical. It consists of swelling and proliferation of the endothelial cells lining the capillaries and smaller veins, accumulation of endothelial leucocytes[3] about the capillaries (Figs. 2, 3, and 5), and active multiplication of these emigrated leucocytes. In addition there is a moderate exudation of serum into the pericapillary tissue (Figs. 1 and 3). In the earlier lesions a very few eosinophils, polymorphonuclear leucocytes, and lymphocytes may also be present in the pericapillary exudate. Diapedesis of red blood corpuscles has not been seen. The endothelial cells of the capillary walls appear swollen; their cytoplasm is finely granular. Occasionally one is seen in mitosis (Fig. 1). The emigrated endothelial leucocytes are young and active. Their nuclei are frequently lobulated. In early cases these leucocytes are in active mitosis (Figs. 1, 2, and 4), as many as three to five mitotic cells sometimes being present about a capillary. Occasionally they

[2] This was always done under anesthesia.

[3] The term endothelial leucocyte is used for the sake of convenience in comparing the lesions in monkeys with the lesions of human measles as recently described by Mallory and Medlar.[5] The authors, however, do not wish to commit themselves to the opinion that the wandering mononuclear phagocytes are derived solely from endothelial cells and not in part from other fixed tissue cells of mesenchymal origin.

show phagocytosis of polymorphonuclear leucocytes and lymphocytes. In later lesions the exudation of serum is less marked, mitotic cells are no longer present, and polymorphonuclear leucocytes are not seen.

These lesions are chiefly in the upper part of the corium and not infrequently lie close to the hair sheaths and sebaceous glands. Although the exudate is primarily pericapillary in location, it also extends into and involves the epithelium of the epidermis, hair sheaths, and sebaceous glands when the capillary lesions lie closely adjacent to these structures.

The lesions in the epidermis (Figs. 6 to 9) are less numerous and conspicuous than those in the corium. In early cases there are minute foci of serous exudate in the epidermis leading to swelling and vacuolation of the epithelial cells of the Malpighian layer (Figs. 6 and 7). The nuclei of these cells are distorted and may appear crescent-shaped and pycnotic. The serum sometimes accumulates under the cornified layer, producing minute vesicles (Figs. 8 and 9). There is slight infiltration of these foci with endothelial leucocytes. Occasionally a more diffuse infiltration of the epidermis with endothelial leucocytes takes place, but this is never very conspicuous. These early exudative lesions quickly give way to retrograde changes. The epithelial cells either singly or in minute clumps show necrosis, the serous exudate disappears, and minute, thickened placques are present in or beneath the cornified layer.

Lesions similar to those in the epidermis are seen in the hair sheaths and in the sebaceous glands when they lie close to a capillary lesion. Infiltration with endothelial leucocytes is apt to be more conspicuous than in the epidermal lesions and may result in the appearance of minute pustules in these structures.

Labial Mucous Membrane.—The lesions in the labial mucous membrane (Figs. 10 to 13) are essentially the same as those occurring in the skin. There are swelling and proliferation of the capillary endothelium (Fig. 10), exudation of serum, and migration of endothelial leucocytes into the pericapillary tissue (Fig. 10), and multiplication of the emigrated leucocytes. A very few polymorphonuclear leucocytes, eosinophils, and lymphocytes are also present in early lesions. Invasion of the epithelium is similar to that of the epidermis, but

usually more marked. It consists of small foci of serum and endothelial leucocytes beneath the stratified epithelium with vacuolation and necrosis of the epithelial cells (Figs. 11 and 12). Diffuse infiltration of the epithelium by endothelial leucocytes is sometimes quite marked. With the progress of the lesion the stratified epithelium covering these minute pustules macerates and sloughs off leaving minute shallow ulcerations on the surface (Fig. 13). At this stage the minute lesions in the epithelium not infrequently show a considerable number of polymorphonuclear leucocytes, presumably due to secondary infection.

Tongue.—The lesions in the mucous membrane of the tongue (Fig. 14) are identical with those in the labial mucosa. The capillaries supplying the papillæ show swelling and proliferation of the endothelium, there are exudation of serum and endothelial leucocytes into the stroma, and invasion of the epithelium by serum and endothelial leucocytes with the formation of minute pustules beneath the stratified layers.

<div align="center">DISCUSSION.</div>

That the symptoms observed in monkeys experimentally inoculated with material containing the virus of measles closely resemble those of human measles seems evident. The incubation period, the conjunctivitis, the enanthem, the exanthem, and the leucopenia closely parallel the similar characteristic features of measles in man. While it is true that the average incubation period in monkeys is 3 to 4 days shorter than it is in man, this might reasonably be expected in view of the presumably large amount of virus inoculated by intratracheal injection. At least it would seem probable that a much larger amount of virus than that which serves to transmit measles from man to man in the natural spread of the disease, is present in 5 to 10 cc. (the amount used) of nasopharyngeal washings from a patient or of tissue emulsion from an infected monkey. If this is so, and there is no evident reason for believing that it is not, the somewhat shorter incubation period is not surprising. The conjunctivitis in monkeys as in man is catarrhal rather than purulent in character, and like that of the human disease it is not infrequently accompanied by definite photophobia and increased lacrimation.

The early, discrete macules on the buccal mucous membrane correspond in time of appearance and in distribution with Koplik spots and differ from them only in the inconstant presence of the characteristic minute, bluish white center. The further evolution of the rash on the buccal mucous membrane is essentially identical with the evolution of the measles enanthem. The exanthem in time of appearance, in the character of the individual lesions, and in evolution likewise closely parallels the exanthem of measles and differs from it only in that it is usually more sparse and less widespread. In fact, there would appear to be only one conspicuous difference between the experimental disease and human measles; namely, the absence of respiratory symptoms in the experimental infection. While this difference cannot be explained at present, it would not appear to constitute a valid reason for rejecting the belief that the reaction observed in experimentally infected monkeys is caused by the virus of measles.

Further and even more conclusive evidence is found in the character of the lesions in the skin and in the buccal mucous membrane. These lesions have been carefully studied in cases of human measles by Ewing[4] and more recently by Mallory and Medlar.[5]

According to the latter authors the lesions in the skin are "due to a proliferative and exudative reaction in and around a small network of capillaries in the upper part of the corium. The reaction consists of occasional mitoses in the lining endothelial cells; of emigration of endothelial leucocytes, and of an active proliferation of them around the vessels; of a very slight emigration of polymorphonuclear leucocytes and lymphocytes; and of phagocytosis of them by the endothelial leucocytes.

"The exudation of serum and endothelial leucocytes at first is active, and passes to the adjoining epidermis, hair sheaths and sebaceous glands. It often collects in small foci, forming minute vesicles and pustules. The epithelial cells involved in the exudation undergo necrosis. By the time the exanthem is clearly evident, these minute lesions in the epidermis are already beginning to dry up, and later desquamate as scales. In the meantime the endothelial leucocytes in the corium continue to proliferate and accumulate around the blood vessels for two to four days, and then gradually disappear.

"Koplik spots correspond exactly to the minute early lesions of the epidermis,

[4] Ewing, J., *J. Infect. Dis.*, 1909, vi, 1.

[5] Mallory, F. B., and Medlar, E. M., *J. Med. Research*, 1920, xli, 327.

but instead of drying up they tend to macerate, and may terminate in erosions or, if secondarily infected, in ulcerations."

It is clear from the foregoing description that the lesions of the skin and buccal mucosa in human measles are exactly like those found in ie skin and buccal mucous membrane of the experimental infection induced in monkeys.[6]

SUMMARY AND CONCLUSIONS.

The symptomatology of the reaction induced in monkeys by inoculation with material containing the virus of measles is described. The symptoms and course of this reaction closely parallel those of human measles.

The microscopic pathology of the lesions of the skin and buccal mucous membrane of monkeys experimentally infected with the virus of measles is also described. These lesions are essentially identical with the corresponding lesions of measles in man.

EXPLANATION OF PLATES.

PLATE 37.

FIG. 1. Early lesion in the corium showing mitosis of an endothelial cell lining a capillary, and pericapillary exudate of serum and endothelial leucocytes, one of which is in mitosis. $\times$ 1,000.

FIG. 2. Endothelial leucocytes accumulated about a capillary in the corium; one is shown in mitosis. $\times$ 1,000.

PLATE 38.

FIG. 3. Early lesion about a small vein in the corium showing an exudate of serum and endothelial leucocytes. $\times$ 1,000.

FIG. 4. Two endothelial leucocytes in mitosis near a capillary in the corium. $\times$ 1,000.

FIG. 5. Exudate of serum and endothelial leucocytes in the corium. $\times$ 500.

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[6] We wish to express our gratitude to Dr. Mallory for being so kind as to examine histological sections of the skin and buccal mucosa from the infected monkeys. We are also indebted to him for permission to quote his opinion that the lesions in the monkeys present essentially the same histological picture that is found in the corresponding lesions of measles in man.

(Blake and Trask: Studies on measles. II)

FACTORS GOVERNING THE EXCRETION RATE OF UREA.*

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In using the rate of urea excretion as criterion of the functional capacity of the kidney, there are two known factors, besides the excretory ability of the kidneys, which influence the urea excretion rate to such an extent that they must be either reduced to constancy, or measured and allowed for by calculation, before the excretion rate itself can be taken as even an approximate measure of the third factor; *viz.*, the functional capacity. Of these two factors, one has been recognized as the concentration of urea in the blood since the publication of the papers of Ambard and his collaborators (Ambard (1910, 1920), Ambard and Papin (1909), Ambard and Weill (1912)), whose work has inspired many investigations, including our own. The other factor was conceived by these authors to be the concentration of urea in the urine, but we believe that we shall demonstrate in this paper that it is rather the rate of volume output of urine.

Effect of Blood Urea Concentration on Excretion Rate.—Ambard held that, other factors being equal, the excretion rate of urea increases as the square of the blood urea concentration; *i.e.*, that doubling the blood urea quadruples the excretion rate. Marshall and Davis (1914), on the other hand, found that when plenty of water is given "the rate of excretion of urea in normal animals is directly proportional to the concentration of urea in the blood." Further data indicating an approximate proportionality between blood urea con-

centration and rate of urea excretion were published by Pepper and
Austin (1915), by Addis and Watanabe (1916), and by Addis, Barnett,
and Shevky (1918).

The conclusions reached in this paper are based chiefly upon data
from human subjects. We have, however, performed a sufficient
number of experiments on dogs to confirm the latter authors. In
some of these experiments we induced marked variation in the blood
urea concentration by administering urea by stomach tube, in others
by feeding an amount of meat equivalent to 5 per cent of the animal's
body weight. The blood samples were drawn from the jugular vein
by puncture through the skin. The results of four experiments are
shown in Table I.

Simple inspection of the ratios in the seventh column shows that
despite wide fluctuations in both D and B, the ratio $\dfrac{D}{B}$ is fairly con-
stant. In Dog 1 only one value out of eleven for the ratio in the
two experiments lies outside the range 57 ± 11, and in the experi-
ments with Dog 2 only one value out of the fifteen lies outside the
range 115 ± 26. Fivefold increase in blood urea is accompanied by
almost exactly fivefold increase in excretion rate.

The percentage variations in the Ambard constant $\dfrac{D\sqrt{C}}{B^2}$ are
much greater than in the simple $\dfrac{D}{B}$ ratio.

The ratio $\dfrac{D}{B}$ appears to be independent of changes in volume out-
put. This independence is only apparent, however. The animals
drank as much water as they wished, and this with the urea formed
or fed induced such a diuresis that practically all the observations
were made when the volume output exceeded the "augmentation
limit" which will be discussed later.

*The Effect of Urine Volume and Concentration on the Excretion
Rate of Urea.*—When volume influences are reduced to a minimum
by keeping the volume output high, our experiments, and those of
Marshall and Davis (1914), indicate that the relationship of the
urea excretion is more nearly expressed by the simple relationship

TABLE I.

Effect of Varying Blood Urea Concentration in Dogs.

Experimental Details.	Time of period.	B, blood urea N.	C, urine urea N.	D, rate of urea N. excretion.	V, rate of urine volume excretion.	D/B	DV√C̄/B² Ambard constant.
		gm. per liter	*gm. per liter*	*gm. per 24 hrs.*	*liter per 24 hrs.*		
Experiment I. Dog 1, 14.5 kilos. 14.5 gm. urea by mouth at 11.49 a.m.	9.47–10.49 a.m.	0.109	12.3	5.8	0.47	53	1,650
	10.49–11.47 "	0 107	6 13	5.7	0.93	53	1,220
	11.47 a.m.–1.49 p.m.	0 504	20.4	25.9	1 27	51	457
	1.49–2.52 p.m.	0 437	22.5	29 5	1 31	67	725
	9.47–11.53 a.m.	0 120	6 75	7.3	1.08	61	1,320
Experiment II. Dog 1, 14.5 kilos. 14.5 gm. urea by mouth at 10.23 a.m.	9 25–10.21 a.m.	0 127	8 54	6 4	0.75	50	1,040
	10 21–11.26 "	0 611	20 8	28.3	1.36	46	342
	11 26 a.m.–12.27 p.m.	0 594	24.6	36 5	1.48	64	517
	12 27–1.30 p.m.	0.497	7.11	33 6	4.73	68	367
	1.30–2.27 "	0.381	2.80	25.5	9 10	67	294
	9 44–11.01 a.m.	0 235	9 44	18 5	1 96	79	1,000
Experiment III. Dog 2, 23.0 kilos. 23 gm. urea by mouth at 10.26 a.m.	10.21–10.56 a.m.	0.256	10 6	26 1	2 46	102	1,200
	10 56–11.26 "	0 686	14 8	63.1	4 27	92	510
	11.26–11.56 "	0 616	19 7	63 3	3 20	103	740
	11.56 a.m.–12.30 p.m.	0 532	20 0	54 9	2 74	103	860
	12.30–1.26 p.m.	0 429	18 5	45.7	2 47	107	1,070
	1 26–2.25 "	0 347	22 6	32 8	1 45	94	1,300
	2 25–3 25 "	0 278	27 6	24.6	0 89	89	1,680
	3 25–4 25 "	0 215	23 2	22 8	0 98	106	2,380
Experiment IV. Dog 2, 23.0 kilos. 1.15 kilos meat at 10.57–11.00 a.m.	10 09–10 56 a.m.	0 092	3 90	11 5	2 94	125	2,690
	12 02–1.05 p.m.	0 134	10 15	14 4	1 42	107	2,550
	1.05–2.05 "	0.142	7 38	28 4	3 85	200	3,800
	2.05–3.05 "	0.227	10 9	30.6	2 81	135	1,960
	3.05–4.05 "	0 266	7.36	30.4	4.13	114	1,160
	4.05–5.00 "	0 268	10 8	37.9	3.51	141	1,720
	9.12–9.57 a.m.	0.132	5 56	12.8	2.30	97	1,740

$\dfrac{D}{B} = K$ than by any more complex function of D and B. Addis found the same to be true with rabbits, especially when other factors were reduced to a relative minimum by making B very large (by feeding large amounts of urea).

Since the $\dfrac{D}{B}$ ratio tends to be constant when variations of other factors lack relative importance, it seemed that a logical way to measure the influence of the other factors on the excretion rate would be to determine their effect on the $\dfrac{D}{B}$ ratio when they attain such importance. For this purpose data from experiments on normal men are available in the papers of McLean (1915) and of Addis and Watanabe (1916), and to these we have added a number by experiments of our own.

In the experiments of Addis and of McLean, and in our own, the urine was collected over short periods, usually 60 or 72 minutes (ours varied in all from $\frac{1}{2}$ to 3 hours), and blood was taken at the middle of each period. The rate of volume output of urine was lowered by avoidance of water and food, or raised by drinking water, or, for large volumes, dilute salt solution (about 0.4 per cent). No attempt was made to regulate activity or general diet. Urea was determined by Marshall's urease method with the technique of Van Slyke and Cullen (1914).

DISCUSSION OF DATA.

In Figs. 1a, 2a, 3a, and 4a, the data on the four individuals from Table II are plotted, values of $\dfrac{D}{B}$ being used as ordinates, values of $\dfrac{1}{C}$ as abscissæ. In Figs. 1b, 2b, 3b, and 4b, we have for comparison on scales as nearly like as possible plotted the values of $\dfrac{D}{B}$ against the values of V. Concerning the nature of the relationships of urea excretion to urine volume and concentration, the results expressed in Table II and in the figures indicate the following:

1. Increase in rate of urine volume excretion up to a certain point, varying between 2.5 and 6 liters per 24 hour time unit in the different individuals, results in a regular increase in the $\dfrac{D}{B}$ ratio. Quantita-
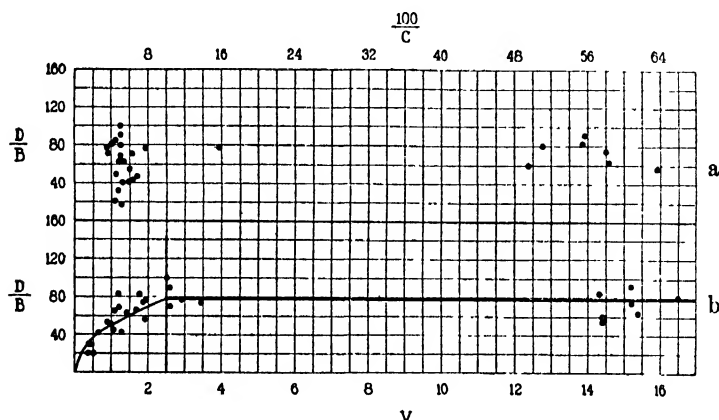
FIG. 1. Data from Addis on Ad.

(a) Upper half: Ordinates $= \dfrac{\text{urea excretion rate}}{\text{blood urea concentration}}$

Abscissæ $=$ urea concentration in urine

(b) Lower half: Ordinates $= \dfrac{\text{urea excretion rate}}{\text{blood urea concentration}}$

Abscissæ $=$ rate of urine volume excretion.
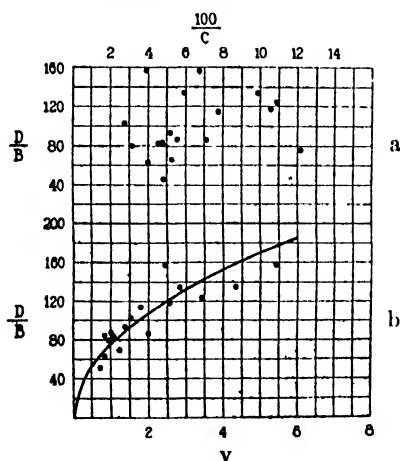
FIG. 2. Data from McLean on himself. Ordinates and abscissæ as in Fig. 1.

tively, this increase is approximately proportional to the square root of the rate of urine volume excretion. This fact is shown by the manner in which the points representing the experimental data follow the curves, which represent the equation $\dfrac{D}{B} = K\sqrt{V}$. It
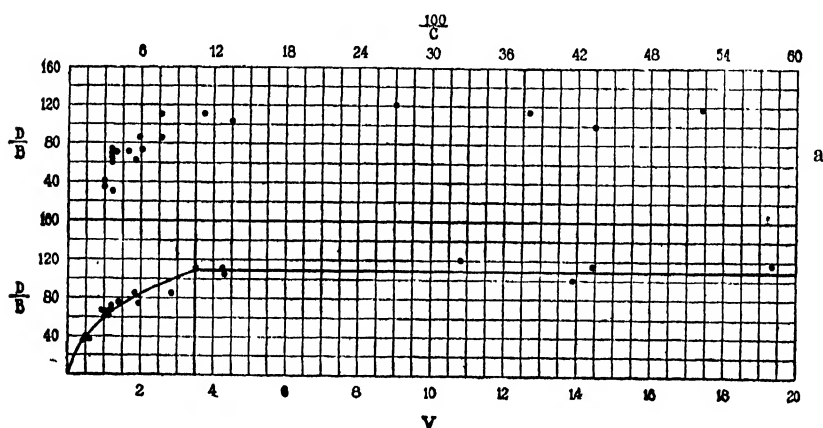
FIG. 3. Data on Austin. Ordinates and abscissae as in Fig. 1.
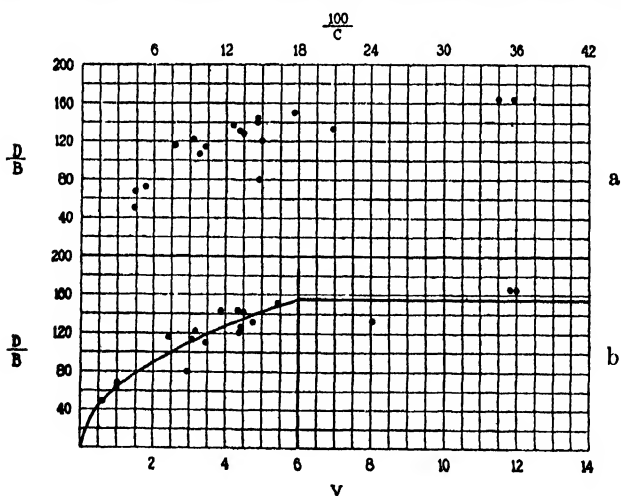
FIG. 4. Data on Van Slyke. Ordinates and abscissæ as in Fig. 1.

does not seem that they would follow so closely curves of any other form. We have tried curves representing other functions of V, such as $V^{\frac{1}{3}}$, $V^{\frac{1}{3}}$ and log V without finding one that so nearly approximates the experimental results.

TABLE II.

Effect of Varying Blood Urea and Volume Output of Urine on the Excretion Rate of Urea in Man.

Subject No.		B, blood urea.	D, rate of urea excretion.	V, rate of urine volume excretion.	C, concentration of urea in urine	$\dfrac{D\sqrt{C}}{B^2}$, Ambard constant.	$\dfrac{D}{B}$	$\dfrac{D}{B\sqrt{V}}$, present constant	$\dfrac{D}{B\sqrt{A}}$
		gm. per liter.	gm. per 24 hrs.	liters per 24 hrs.	gm per liter.				
Ad.	1	0.450	9.5	0.41	23 3	226	21 1	33 0	
	2	0.300	8.9	0.43	20 6	448	29.7	45.3	
	3	0.495	9.4	0.48	19.5	170	19 0	27 4	
	4	0 276	11.5	0.67	17 1	365	41.7	51 0	
	5	0.442	23.1	0.94	24 6	588	52.3	53.9	
	6	0.427	21.8	0 98	22.3	564	51 1	51.5	
	7	0.322	15.1	1.05	14.4	553	46 9	45 7	
	8	0.322	20.6	1.08	19.1	870	64 0	61.6	
	9	0.367	25.0	1 22	20.5	843	68.1	61 7	
	10	0 346	28.7	1.22	23 5	1,160	82.9	75.1	
	11	0.472	20.1	1.27	15 8	358	42.6	37 9	
	12	0.465	28.9	1.44	20 1	448	62.2	51 9	
	13	0.607	45 4	1.66	27.5	504	74.8	56 8	
	14	0.495	40 7	1 71	23 8	810	82.2	62.9	
	15	0.682	52.0	1 87	27.8	592	76 2	55 8	
	16	0.540	30 4	1.89	16 3	420	56 3	41 0	
	17	0 322	24.7	1 92	12 9	854	76.7	55 3	
	18	0.495	49 8	2.50	19 9	904	100 6	63 7	
	19	0 585	40 9	2 57	15.9	484	69 9	43 6	44 2*
	20	0.577	51.6	2.62	19.7	688	89 4	55 3	56.6
	21	0.765	59.7	2.90	20 6	462	78 0	45 8	49.3
	22	0.300	22.1	3.46	6 40	672	73 7	39 6	46.6
	23	0.312	25.9	14 3	1 81	358	83.0	22 0	52 5
	24	0.402	22.6	14.4	1.57	175	56 2	14 8	35 5
	25	0.489	29.1	14.4	2 02	173	59.5	15.7	37.6
	26	0.300	27.4	15 2	1 80	408	91 3	23 4	57.8
	27	0.354	26.2	15 2	1.72	274	74 0	19 0	46 8
	28	0.414	26.2	15 3	1.71	200	63 3	15 3	40.1
	29	0.408	32.4	16.5	1.96	273	79.4	19 5	50.3
Mc.L.	1	0.299	14.7	0 72	20.7	750	49 2	58 0	
	2	0 321	20.1	0.82	24.8	980	62 6	69 0	
	3	0.214	18.0	0.86	21.0	1,810	84 1	90 0	
	4	0.363	29.3	0 96	31.2	1,250	80 7	84 0	
	5	0.221	19.4	1.07	18.1	1,690	87 8	85 0	
	6	0.298	25 0	1.12	22 3	1,330	83 9	75.0	
	7	0.341	23.2	1.24	18.7	860	68.0	61.0	
	8	0.286	26 5	1.38	19.1	1,420	92.7	79 0	
	9	0.513	52.6	1.54	35 1	1,150	102.5	82.0	
	10	0.206	23.0	1.76	13 1	1,960	111.7	84.0	
	11	0.321	27.8	2.00	13 9	1,010	86.6	61.0	

*The value employed for augmentation limit (A) is for Ad. 2.5; J. H. A. 3.5; V. S. 6.0.

TABLE II—*Concluded.*

Subject No		B, blood urea.	D, rate of urea excretion.	V, rate of urine volume excretion.	C, concentration of urea in urine.	$\frac{D\sqrt{C}}{B^2}$, Ambard constant.	$\frac{D}{B}$	$\frac{D}{B\sqrt{V}}$, present constant.	$\frac{D}{B\sqrt{A}}$
		gm per liter	gm per 24 hrs	liters per 24 hrs.	gm per liter				
	12	0 406	63 3	2 42	25 2	1,930	155 9	100 0	
	13	0 206	24 3	2.56	9 5	1,760	118.0	74 0	
	14	0 211	28 2	2 80	10 1	2,020	133 6	80 0	
	15	0 254	31 2	3 40	9 2	1,470	122 8	73.0	
	16	0.542	72 5	4 32	16 7	1,010	133 8	64 0	
	17	0.539	84 7	5 40	15 7	1,150	157.1	68.0	
H. A.	1	0.424	15.6	0 47	33 0	500	36 8	54 0	
	2	0.443	17 7	0 51	32 7	520	40 0	56.0	
	3	0 360	14 3	0 53	28.0	631	39 7	54 5	
	4	0.445	29 4	0 97	30 4	818	66 1	67 1	
	5	0.450	27 6	1 00	27 6	716	61 3	61 4	
	6	0 442	28 7	1 04	27.7	780	64 9	64 0	
	7	0 313	19 6	1 09	18 0	848	62.6	59 9	
	8	0 448	30 8	1 15	26 8	794	68 8	64 1	
	9	0.452	32 2	1 19	27.6	820	71 2	65 0	
	10	0.371	27 0	1 32	20 2	882	72 8	63 4	
	11	0 360	31 1	1 81	17 2	995	86 4	64 2	
	12	0 425	31 7	1 94	16 4	710	74 6	53 0	
	13	0 422	36 2	2 77	13 1	740	85.8	52.0	
	14	0 423	46 4	3 53	13 1	940	109 7	58 5	58 8*
	15	0 340	37 7	4 24	8.9	972	110 9	53 8	59 3
	16	0 313	32 7	4 30	7 6	921	104 5	50 4	55 8
	17	0 350	42 3	10 80	3 9	680	120 9	36 8	64 6
	18	0 320	31 6	13.90	2 3	470	98 8	26 6	52 9
	19	0 332	38 0	14 45	2 63	559	114.5	30 2	61 1
	20	0 323	37 9	19.30	1.91	502	117.3	26 7	62.7
V. S.	1	0.301	15 0	0 65	22 9	800	59 8	62.0	
	2	0 355	24 2	1 03	23 3	930	68.2	61 0	
	3	0.300	21.5	1.13	19 0	1,040	71.7	67.5	
	4	0.269	31 2	2 46	12 8	1,540	116 0	74.0	
	5	0.243	19 7	2 90	6 8	870	81.1	47.6	
	6	0.268	30 7	3 12	9 8	1,590	114 6	63 0	
	7	0.283	34 5	3.19	10 8	1,415	121.9	68 3	
	8	0.326	35 6	3 46	10 3	1,075	109.2	58.7	
	9	0 209	28 8	3.64	7 9	1,853	137.8	72.3	
	10	0.213	30.2	4 31	7 0	1,760	141.8	68.3	
	11	0 243	29 5	4 40	6 7	1,292	121 3	57.9	
	12	0 255	32 8	4 42	7 4	1,372	128 6	61 2	
	13	0.219	31.4	4 48	7 0	1,730	143 4	67.7	
	14	0.276	36.5	4.74	7 7	1,328	132 2	60.7	
	15	0.206	31 2	5 47	5 7	1,755	151.5	64.7	
	16	0.285	38.6	8 05	4.8	1,040	135 4	47.7	55.1*
	17	0.204	34.2	11.80	2.9	1,398	167 6	48.8	68.4
	18	0.202	33.6	12.00	2.8	1,376	166.3	48.0	67.9

2. It is evident from Figs. 1*b*, 3*b*, and 4*b*, that a limit is reached as the rate of urine volume excretion increases, beyond which further increase in the rate of urine volume excretion has no further effect in augmenting the $\dfrac{D}{B}$ ratio. This limit we will call the augmentation limit. Of the existence of this limit there seems no room for doubt after inspection of the figures named, especially Fig. 1. The variation of individual observations from the mean curve is sufficient, however, to make the exact location of the limit in a given individual somewhat difficult. The method we have employed is to calculate the constant, $\dfrac{D}{B\sqrt{V}} = K$, for all observations on a given individual, arranging them in the order of increasing values of V as in Table II. Inspection of the series of values for K shows them to lie within a certain range until for the higher values of V they decrease progressively. Omitting these progressively decreasing values, the mean of the remainder is determined and the curve shown in the figures is that obtained by the use of this mean as K in the equation $\dfrac{D}{B} = K\sqrt{V}$. A horizontal line is then drawn through the mean position of the observations at the higher values of V. The value of V at the intersection of this horizontal line with the curve is considered to be the augmentation limit for the individual. The value of V at this point is designated as A and is used in place of V in calculating the constant for the individual in observations in which the urine volume excretion exceeds the augmentation limit. The values of the constant thus calculated are shown in the last column at the right of Table II.

In this manner we have estimated the approximate augmentation limits of the four individuals studied as follows:

Ad............................ ...	Rate of 2.5 liters per 24 hrs.
Austin...........................	" " 3.5 " " 24 "
McLean..........................	" " over 5 liters per 24 hrs.
Van Slyke.......................	" " 6.0 liters per 24 hrs.

It is probably because many of Addis' observations were made at rates of urine volume excretion exceeding the augmentation limit that he concluded that the rate of urine volume excretion is without effect on the rate of urea excretion. In man, in the limited number of observations that we have considered, the augmentation limit lies between the rates 2.5 and 6 liters of urine volume excretion per 24 hours, hence is in excess of the rate of urine volume excretion usually observed except after the drinking of large amounts of fluid.

3. The concentration of urea in the urine was believed by Ambard to be a factor of definite influence upon the urea excretion rate. We
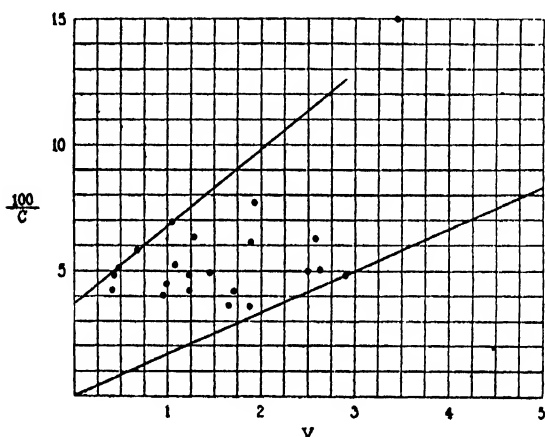
FIG. 5. Data from Addis on Ad.

Ordinates = reciprocal of concentration of urea in urine
Abscissæ = rate of urine volume excretion

have tested this probability by attempting to plot in Figs. 1a, 2a, 3a, and 4a, curves showing the relationship of C, the concentration of urea in the urine, to the $\dfrac{D}{B}$ ratio. When these figures are inspected no such effect is to be seen in two of the four subjects (Ad. and Mc-Lean). In the other two (Austin and Van Slyke) the $\dfrac{1}{C}$ points follow curves similar to those followed by the V points, but follow them less closely (calculation shows the average deviation to be twice as great). The apparent relationship between excretion ratio and
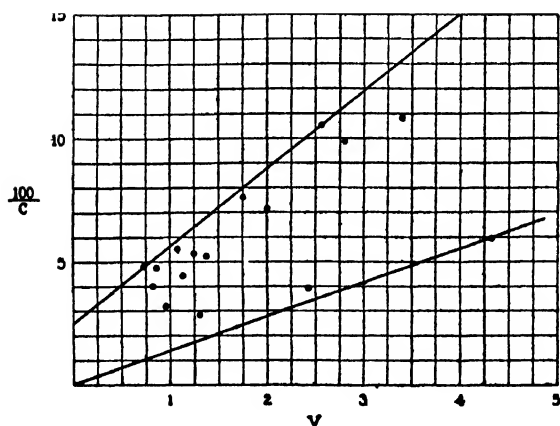
Fig. 6. Data from McLean on himself. Ordinates and abscissæ same as Fig. 5.
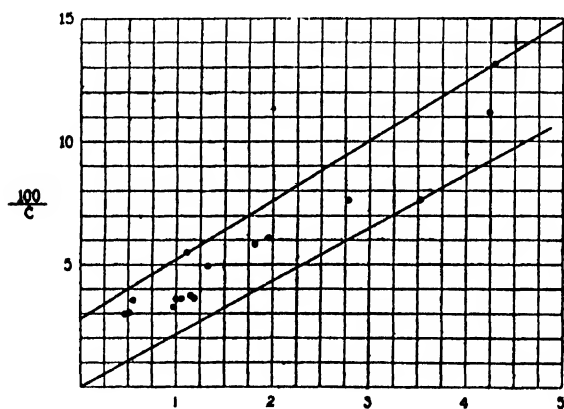
Fig. 7. Data on Austin. Ordinates and abscissæ same as Fig. 5.
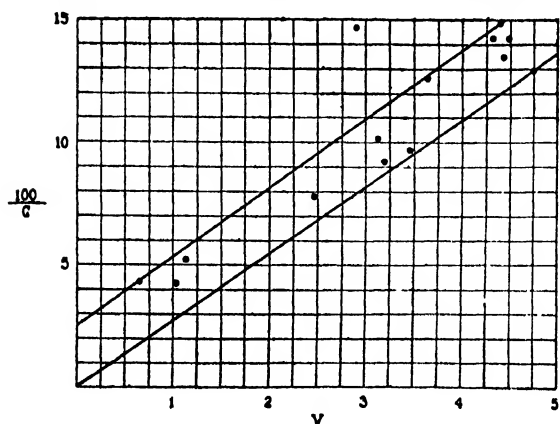
Fig. 8. Data on Van Slyke. Ordinates and abscissæ same as Fig. 5.

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urinary concentration in these two subjects may be due to the fact that in all the experiments performed on them the concentration of urea in the urine varies quite consistently in inverse proportion to the volume, so that the urinary concentration happens to be an indirect measure of urinary volume. The presumable cause of this consistency is that in these experiments the changes in rate of excretion were induced by varying the fluid intake. In some of the experiments of Addis and McLean, great variations in the blood urea were also induced, resulting in urines that were high both in volume and concentration (see Figs. 5, 6, 7, and 8). In these cases, where concentration fails to be an indirect measure of volume, it also fails to show any relationship whatever to rate of urea excretion. The conclusion seems with a considerable degree of probability to be true that C is related to $\dfrac{D}{B}$ only when it happens to approximate inverse proportionality to V.

Equations Expressing Observed Relationships.

The above relationships of urea excretion rate to (1) blood urea and (2) urine volume, are expressed in the following equations:

$$(1) \quad D = K_1 B$$

$$(2) \quad D = K_2 \sqrt{V}$$

Expressing both relationships in one equation, we have

$$(3) \quad D = KB \sqrt{V}$$

For individuals of varying body weight, an allowance must be made for this factor. For this purpose it appeared most logical, and was found to give the most consistent results, to express the excretion rates of both urea and urine volume excretion on a per kilo basis, as follows:

$$(4) \quad \frac{D}{W} = KB \sqrt{\frac{V}{W}} \quad \text{or}$$

$$(5) \quad D = KB \sqrt{VW} \quad \text{or}$$

$$(6) \quad K = \frac{D}{B \sqrt{VW}}$$

$K = 7.5 \pm 3$ for normal individuals, as will be shown.

When V exceeds the augmentation limit, normally 2.5 to 6 liters per 24 hours, which we will designate as A, the equation becomes

$$(7) \quad K = \frac{D}{B\sqrt{AW}}$$

In Fig. 9 are given all the data on all individuals taken from the published figures of McLean and of Addis and Watanabe, and from our own observations that have values for V below a mean augmentation limit of 3.5 liters per 24 hours, and plotted in accordance with
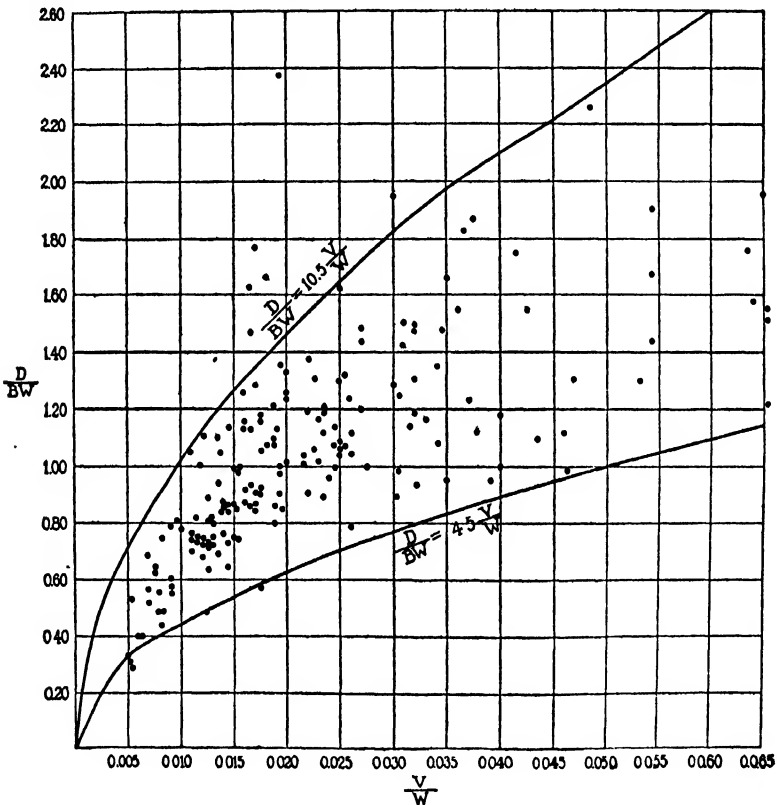
FIG. 9. Showing all observations on normal individuals with urine excretion rates below augmentation limit, reported by Addis, by McLean, and by ourselves.

$$\text{Ordinates} = \frac{\text{rate of urea excretion}}{\text{blood urea concentration} \times \text{body weight}}$$

$$\text{Abscissæ} = \frac{\text{rate of urine volume excretion}}{\text{body weight}}$$

this formula, values of $\dfrac{D}{BW}$ being plotted as ordinates, those of $\dfrac{V}{W}$ as abscissæ. It is evident that the data arrange themselves along the curve representing the equation $\dfrac{D}{BW} = K\sqrt{\dfrac{V}{W}}$ which is identical with $K = \dfrac{D}{B\sqrt{VW}}$. K having a value of 7.5 ± 3.

The lower curve represents values for $K = 4.5$, the upper for $K = 10.5$. About 96 per cent of the determinations fall within these limits. Whether the 4 per cent that fall outside are due to unnoted analytical errors, or to occasional failure to empty the bladder completely at one end of the period, or whether they represent the actual frequency with which unrecognized factors deflect the excretion rate outside the usual range, it is at present impossible to say.

The greater part of the determinations yield results in the range midway between the two curves, covered by values of K between 6 and 9.

Variability of Results Obtained with Normal Individuals.

The fact that the "urea secretory constant" calculated even by the present formula, $K = \dfrac{D}{B\sqrt{VW}}$ varies in normal individuals between limits as wide as 7.5 ± 3 indicates that the two factors, rate of urine volume excretion and blood urea concentration, are not the only ones aside from kidney excretory ability which govern the rate of urea output. The variation in the constant indicates the presence of other factors, as yet unrecognized, perhaps of nervous or chemical nature, which influence to a minor, but at times marked, degree the rate of urea excretion. If these factors, or any one of them, can be both recognized and measured, we shall have advanced another step in the accuracy of our knowledge of renal function.

Relationships of Present Formula to That of Ambard.

1. The First Law of Ambard.—According to the first law of Ambard, with constant concentration of urea in the urine, the urea output varies as the square of the blood urea concentration.

$$\frac{D}{B^2} = K$$

The relation between the first law of Ambard and the present formula becomes clear, if the conditions of the experiments employed by Ambard in arriving at his first law are considered. These experiments were conducted by placing dogs on a diet of meat and withholding all water other than that present in the meat. Under such conditions Ambard observed that the dog secretes a urine of constant and maximal urea concentration and more or less of both urine and urea per time unit according as the blood urea concentration is increased or decreased by the amount of meat fed.

If the present formula be applied to the special case in which the urea concentration in the urine, C, remains constant, the following is derived:

$$C = k_1 = \frac{D}{V}$$

hence

$$\frac{D}{k_1} = V \text{ or } k_2 D = V$$

substituting in

$$\frac{D}{B \sqrt{V}} = K$$

we have

$$\frac{D}{\sqrt{k_2 D}} = KB$$

combining constants

$$\frac{D}{\sqrt{D}} = k_3 B$$

or

$$\sqrt{D} = k_3 B \text{ or } \frac{D}{B^2} = k_4$$

$\dfrac{D}{B^2} = K$ is the mathematical expression of Ambard's first law.   Therefore, Ambard's first law is a special case of our general formula limited to the condition which obtains when the concentration of urea in the urine is maintained constant.

2. *The Second Law of Ambard.*—According to the second law of Ambard, when the concentration of urea in the blood is constant, the rate of excretion of urea is inversely proportional to the square root of the urea concentration in the urine.

$$D \sqrt{C} = K$$

or

$$D = \frac{K}{\sqrt{C}}$$

If our general formula $\dfrac{D}{B \sqrt{V}} = K$ is true, however, the following relations may be derived:

$$(1) \quad \frac{D}{B \sqrt{V}} = k$$

$$(2) \quad \frac{D \sqrt{D}}{B \sqrt{V} \sqrt{D}} = k$$

since

$$(3) \quad \frac{\sqrt{D}}{\sqrt{V}} = \sqrt{C}, \qquad \frac{D \sqrt{C}}{B \sqrt{D}} = k$$

or

$$(4) \quad D \sqrt{C} = kB \sqrt{D}$$

Whence $D\sqrt{C} = K$ not when B is constant, as Ambard's second law states, but when $B\sqrt{D}$ is constant or when $B = \dfrac{K}{\sqrt{D}}$

Hence Ambard's second law is inconsistent with our formula.

The Combined Laws of Ambard.

The quantitative differences between the present equation and that conceived by Ambard become clearer when the urine concentration, C, of the Ambard equation is transformed into terms of

volume by substituting $\dfrac{D}{V}$ for C.   The equations may be compared by arranging them both to express values of D.   The complete Ambard equation in this form becomes

$$D = \frac{KB^2W}{\sqrt{\dfrac{D}{V}}}$$

whence

$$D^{\frac{3}{2}} = KB^2V^{\frac{1}{2}}W$$
$$D = K'B^{\frac{4}{3}}V^{\frac{1}{3}}W^{\frac{2}{3}} \quad \text{(Ambard equation)}$$

According to the equation developed in this paper

$$D = KBV^{\frac{1}{2}}W^{\frac{1}{2}} \quad \text{(present equation)}$$

The greater accuracy with which the present equation, as compared with that of Ambard, appears to express the relationships observed in man is indicated by Table III, which is a summary of the data presented in Table II.

In order to permit accurate comparison of the constants, we have expressed both Ambard's and ours in such form that D appears in the first power in the numerator of each.   Thus Ambard's constant $K = \dfrac{B}{\sqrt{\dfrac{D}{W}}\sqrt{C}}$ we have inverted and squared giving it the form $\dfrac{D\sqrt{C}}{B^2W} = K$ or for comparison of repeated observations on the same individual simply $\dfrac{D\sqrt{C}}{B^2} = K$.   Table III shows that in three of the four individuals studied the mean percentage deviation from the average urea secretory constant for the individual, calculated by the equation derived in this paper, viz. $K = \dfrac{D}{B\sqrt{V}}$ is approximately half as great as the percentage deviation in the Ambard quotient for the same individual.   In the fourth individual it is only one-third as great.

Also, when the weight factor is introduced for each individual by taking the average of all the constants determined on the indi-

vidual and correcting it by his body weight, the average constants thus determined by the present equation $K = \dfrac{D}{B\sqrt{VW}}$ vary, in the four individuals, between the limits 6.1 and 8.5. The average constants calculated by Ambard's equation $K = \dfrac{D\sqrt{C}}{B^2W}$ vary from 8.1 to 18 for the four subjects. While the number of subjects is too small to base conclusions on this difference, the latter is so marked that it may be significant.

TABLE III.

Summary of Data of Table II (below Augmentation Limit).

Subject.	Weight.	No. of determinations.	$\frac{D\sqrt{C}}{B^2}$			Mean deviation from average.		$\frac{D\sqrt{C}}{B^2W}$	$\frac{D}{B\sqrt{V}}$			Mean deviation from average.		$\frac{D}{B\sqrt{VW}}$
			Minimum.	Maximum.	Average.	Numerical value	Per cent of average.	Average.	Minimum.	Maximum.	Average.	Numerical value.	Per cent of average.	Average.
	kilos													
Ad.	73	18	170	1,160	594	209	35	8.1	27	75	52	9.0	17.3	6.1
McL.	77	17	750	2,020	1,390	350	25	18.0	58	100	76	9.2	12.1	8.5
J. H. A.	66	13	500	995	750	105	14	11.3	52	67	60	4.6	7.7	7.4
V. S.	72	15	800	1,853	1,356	288	21	17.9	48	74	64	5.0	7.8	7.5

We believe that by directing our attention to urinary volume as well as concentration, and by utilizing data obtained with methods for urea determination perhaps more accurate than those originally available to Ambard, we have been able to express the influence of the most important blood factor (urea concentration) and the most important urinary factor (rate of urine volume excretion), on the rate of urea excretion in an equation of definitely greater accuracy than that originally conceived by Ambard. If this belief is confirmed, the present work is to be regarded not as a disproof of Ambard's, but rather as an advance which has proceeded along the path opened by his researches, and which has resulted in a somewhat closer approximation to his ideal of accurate functional measurement.

Use of the Creatinine Ratio in Calculating Excretion Rates.

It has been our experience that in determining the urea excretion rate, the most frequent source of error is failure to secure complete emptying of the bladder at the beginning or end of the period of collection of urine. This difficulty is more likely to be encountered in patients than in normal subjects. When the time elapsing between the two emptyings of the bladder is employed for the calculation of

TABLE IV.

Excretion Constants in Normal Subjects Calculated by Time and Creatinine Ratios Respectively.

Subject.	Length of period.	Volume urine.	Day's volume calculated by		Urine urea No.	Day's urea N calculated by		Blood urea N	Urea index calculated by	
			Time.	Creatinine.		Time.	Creatinine.		Time.	Creatinine.
	min.	*cc.*	*liters*	*liters*	*per cent*	*gm.*	*gm.*	*percent*	$\dfrac{10\,D}{B\sqrt[3]{VW}}$	$\dfrac{10\,D}{B\sqrt[3]{VW}}$
J. H. A.										
1	60	20	0 48	0.47	15 4	7 4	7.3	0.198	66	66
2	63	22	0 50	0.51	16 3	8.2	8 2	0.207	69	69
6	59	41	1.00	1.04	12.9	12 9	13 4	0.206	80	78
9	63	49	1.12	1.19	12.6	14.1	15.0	0.211	78	80
10	41	39	1.37	1.32	9.5	13.1	12.6	0.173	80	78
12	195	302	2.23	1.94	7.6	17 0	14.8	0.198	71	66
13	53	130	3 53	2.77	6.1	21.6	16.9	0.197	72	63
V. S.										
1	42	31	0.85	0.65	10.7	9 1	7.0	0.140	83	73
2	60	42	1.01	1.13	8.9	9.0	10.0	0.140	75	79
3	42	31	1.06	1.03	10 9	10.8	11.3	0.166	75	79
4	60	102	2.45	2.46	6.0	14.6	14.7	0.126	87	88
6	39	102	3.77	3.12	4.6	17.3	14.3	0.125	84	76
9	30	80	3.85	3.64	3.7	14.2	13.5	0 098	87	85
11	60	184	4.40	4.40	3.1	13.7	13 7	0 113	68	68

D and of *V*, as has been the custom in the use of Ambard's formula, incomplete emptying of the bladder introduces an error which may be very large.

It has been established by Van Hoogenhuyze and Verploegh (1905) and by Shaffer (1908–09) that the hourly elimination of creatinine throughout the day in a given individual is approximately constant.

Taking advantage of this constancy of creatinine excretion, we have found that often more consistent results were obtained when the calculations of D and V were based on the ratio of the creatinine in the sample of urine collected to the 24 hour excretion of creatinine, than when they were based on the ratio of the time interval of the collection period to 24 hours.   The calculation based on the creatinine ratio is made as follows:

$$V = \frac{\text{gm. creatinine excreted per 24 hrs.}}{\text{gm. creatinine per liter in sample of urine secured}}$$
$$D = V \times \text{gm. urea per liter of urine}$$

In entirely normal individuals, nearly identical results are obtained by both methods of calculation (Table IV).

Clinical Application of the Index of Urea Excretion.

The work presented in this paper has been undertaken with the frank intention of obtaining knowledge of normal urea secretion in order that pathological deviations therefrom may be recognized and studied with additional accuracy.   Before application of the results to routine diagnosis can appear justified, however, it is necessary to compare the relationships of urea excretion to its governing factors as established in normal individuals with the relationships found in a sufficient number of patients under the most complete and prolonged clinical observation.   The results of such comparison may be expected to indicate the possible variety of interpretations, diagnostic and prognostic, that may attach to observed abnormalities in the excretion.   Until this has been accomplished, the functional index must be regarded as an object of investigation, rather than as an aid in the clinic.

CONCLUSIONS.

1. The rate of urea excretion per unit of body weight in a normal dog or man increases approximately (*a*) in simple direct proportion to the blood urea concentration, and (*b*) in proportion to the square root of the rate of volume output of urine per unit of body weight, as long as the volume rate remains within ordinary limits.

2. The increase in rate of urea excretion with volume output of urine holds, however, only up to a certain limit of volume output. This "augmentation limit" varied in different normal individuals between 2.5 and 6 liters per 24 hour time unit. Beyond this limit, rise of urine volume to any height does not further accelerate urea excretion.

3. That an augmentation limit exists not only in volume output of urine, but also in blood urea concentration is indicated by the results of Addis, who found that after the blood urea in rabbits reached 2.5 gm. per liter further increase no longer accelerated excretion. Unlike the augmentation limit of urine volume excretion, however, that of blood urea concentration lies at a height so greatly above that ever approximated in normal individuals that it may be neglected in formulating naturally occurring relationships.

4. The observed relationships are expressed in the equation

$$\frac{D}{W} = KB \sqrt{\frac{V}{W}}$$

or

$$K = \frac{D}{B \sqrt{VW}} = 7.5 \pm 3 \text{ (for normal man)}$$

D being the urea output (gm. per 24 hour time unit), B the blood urea (gm. per liter), V the volume output (liters per 24 hour time unit), W the body weight (kilos), and K the excretory constant. For values of V above the augmentation limit, the value of the augmentation limit A replaces V in the formula.

Values for K below the minimum normal limit of 4.5 indicate that for the blood urea concentration, urine volume output, and body weight of the individual, urea excretion is abnormally slow.

5. Ambard's first law, $\frac{D}{B^2} = K$ when C is constant, is shown to be a special case of the above equation. His second law, $D\sqrt{C} = K$ when B is constant, is shown to be inconsistent with our formula.

The greater constancy of results calculated by the equation developed in this paper indicates the probability that it expresses the influence of the chief factors governing excretion with a closer degree of accuracy than does Ambard's equation.

6. The use of a creatinine ratio rather than a time ratio for calculating urinary excretion rates is described, and is advocated when, because of retention in the bladder or of other factors, the accuracy with which the time intervals of the collection period can be measured is doubtful.

7. The physiological studies presented in this paper afford a basis for detecting abnormalities in urea excretion, but not for interpreting the significance of such abnormalities.

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STREPTOCOCCI OCCURRING IN SOUR MILK.

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(Received for publication, June 24, 1920.)

Considerable confusion seems to exist regarding the specific cultural characters of the group of organisms which sour milk. Many have noted the presence of enormous numbers of streptococcic forms in sour milk. The morphology of such bacteria has been studied in detail by Grotenfeld,[1] Günther and Thierfelder,[2] Kruse,[3] and others. Kruse has given the name of *Streptococcus lacticus* to the organism most frequently found and called attention to its resemblance to the pneumococcus. Many, however, disagree with Kruse and place it in the genus *Bacterium*; such names as *Bacterium lacticus*, *Bacterium güntheri*, and *Bacillus lactis acidi* are frequently met with.

In addition to the confusion in nomenclature, one frequently finds reference to the occurrence of *Streptococcus lacticus* in the udder, feces, and saliva, or on the skin of cows. Furthermore, many believe that the milk-souring type is indistinguishable in most respects from the pyogenic type.

Ruediger[4] studied streptococci isolated from milk freshly drawn into milk pails and sterile bottles. Of 32 strains grown in blood agar plates, 8 produced considerable hemolysis about the colonies, 14 were faintly hemolytic, 9 produced green zones, and 1 was non-hemolytic. All the organisms were cocci arranged in chains; rod-like elements were not observed. Ruediger considered that the eight hemolyzing strains were *Streptococcus pyogenes* and the other faintly hemolyzing and green-producing organisms belonged to the *Streptococcus lacticus* group. Further evidence to warrant this assumption is not submitted.

Heinemann[5] states that the group comprises two species,—a bacterium, *B. aerogenes* var. *lacticus* and *Streptococcus lacticus*. It is stated that *Streptococcus lacticus* agrees in morphological and cultural characters with the human pathogenic, sewage and fecal streptococci. He believes that the milk-souring streptococci can be detected on the external surfaces and in the feces of cows as well as

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in the milk during all stages of handling. In a later paper Heinemann[6] published the results of some experiments with two strains of streptococci, one isolated from certified milk, the other from ice-cream. By the passage of these cultures through a series of rabbits and guinea pigs their virulence was increased. In addition, the organisms grew feebly. They produced much less acid in dextrose and lost the power to ferment saccharose, mannitol, and salicin.

Conn, Esten, and Stocking[7] described *Streptococcus lacticus* as a short chained, Gram-positive coccus, which grew on agar as an exceedingly delicate film. Gelatin colonies were characteristic but the medium was not liquefied. Dextrose, lactose, and saccharose were fermented without gas formation. Milk was coagulated. They found these organisms in great numbers in sour milk and state that they frequently made up 99 per cent of the flora.

Shippen[8] in studying the microorganisms of milk in Baltimore referred to *Streptococcus lacticus* as *Bacterium güntheri*. It was the most common type observed by him in sour milk but its presence was also noted in fresh (unsoured) milk. Like Kruse, he stated that the forms varied from short slender rods to distinct coccus-like forms. Many strains closely resembled streptococci when grown in liquid media, but search always revealed some rod-like forms. In these cultures the elongated elements predominated but cocci could also be found. He considers that the sour milk organisms differ from *Streptococcus pyogenes* in several respects; namely, in form, in their inability to hemolyze, their ability to grow at lower temperatures, their lack of pathogenicity for laboratory animals, and their high thermal death-point (65–70°C.).

McGuire[9] considers that *Streptococcus lacticus* is a normal inhabitant of cow feces. Apparently the species was identified largely by morphology and the points noted by Shippen.

Weigmann[10] divides the milk streptococci into two groups: the *Streptococcus mastitidis* Guillebeau group characterized by the coagulation of milk and the formation of gas from carbohydrates; and the lactic acid group, *Streptococcus lacticus*, or *Streptococcus güntheri*. The latter class fails to produce gas. It is surprising to note the frequency with which the mastitis streptococci are referred to as gas-producing organisms throughout the literature.

Sherman and Albus[11] have shown that udder streptococci differ from those found in sour milk. They isolated and studied 50 strains from sour milk and as

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[10] Weigmann, H., in Sommerfeld, P., Handbuch der Milchkunde, Wiesbaden, 1909, 328.

[11] Sherman, J. M., and Albus, W. R., *J. Bact.*, 1918, iii, 153.

many from milk drawn directly from the udder into sterile bottles. The two classes of organisms were studied in groups. It was found that the individual elements of the sour milk type were elongated and grouped most frequently in pairs, but chain formation was common. The udder streptococci were spherical and grew as chains. The action of the two groups in the various media was noted. Thus 40 per cent of the lactic acid cocci fermented mannitol and a like proportion salicin. Of the udder type 76 per cent fermented saccharose and 16 per cent produced acid in salicin; all failed to ferment mannitol. They found that the milk-souring organisms were able to multiply at a temperature as low as 10°C. The udder streptococci grew better at 38°C. but 82 per cent were able to grow at 43°C. Considerable stress is laid on the reducing properties of the lactic acid organisms. The lactic acid types were able to grow in milk containing methylene blue and other dyes and were able to reduce the substances. The udder streptococci failed to grow in the methylene blue medium. In milk containing litmus members of both groups reduced the substance, but the lactic acid streptococci reduced litmus before coagulation, the others after the medium had coagulated.

It will be noted from the foregoing brief résumé that the specific identity of the so called *Streptococcus lacticus* is more or less obscure. The work of Sherman and Albus did much to show that differences existed between the lactic acid and udder groups.

It was considered advisable to study the species or groups of species with the idea that there exists a clear-cut differentiation from the udder or mastitis streptococci. With this in view, milk from various sources was permitted to sour at room temperature and in the incubator. Plate cultures were made from the milk at various times. The isolated organisms were studied in the same type of medium employed in the study of mastitis and equine streptococci.

<div align="center">EXPERIMENTAL.</div>

The following experiment may be regarded as a type. Bottled market milk was purchased and after mixing distributed into sterile wide mouth bottles. These were permitted to stand at room temperature. The milk was examined at various intervals; usually an unopened bottle was chosen each time. A sample of the fresh milk was plated at once; subcultures were made from colonies resembling streptococci. In Table I the protocols of five samples of milk are given.

TABLE I.

Sample.	Length of time left at room temperature. (days)	Acidity of whole milk. (per cent)	Acidity of whey. Titration. (per cent)	Acidity of whey. Hydrogen ion concentration. (pH)	No. of organisms per cc.	Proportion of streptococci.	Subcultures referred to in Table II.
A	0	1.3			2,160	5 per cent.	1, hemolytic. 3, "
A	1	1.5	6.0	4.7	6,300,000	5 " "	4 A, non-hemolytic. 7, hemolytic.
A	2	7.5	7.3	4.6	220,000,000	90 " "	13, " 15, non-hemolytic.
A	3	9.2			107,000,000	2 per cent hemolytic. 90 per cent.	16, " 18, "
A	4	8.4	6.9	4.4	720,000,000	90 " "	18 A, " 20, hemolytic.
A	7	9.0	7.2	4.7	760,000,000	1 per cent hemolytic. 90 per cent.	21, non-hemolytic. 21 A, "
A	14	8.1	7.4	4.8	960,000,000	85 " "	23, " 25, "
B	0				7,500	5 per cent hemolytic.	25 A, " 27, hemolytic.
B	1	5.3	4.2	5.3	12,800,000	90 per cent.	28, " 29 A, non-hemolytic.
B	2	9.1	6.8	4.8	3,500,000,000	1 per cent hemolytic. 95 per cent. Few hemolytic colonies.	30, " 30 A, "

B	3	9.6	7.7	4.6	5,000,000,000	90 per cent non-hemolytic.	31, non-hemolytic. 32 A, "
B	5	7.5	7.0	4.9	3,000,000,000	90 " " " " Less than 1 per cent hemo-lytic.	33, " 34, hemolytic.
B	7	9.0	8.6	4.8	1,500,000,000	95 per cent non-hemolytic. 1 " " hemolytic.	35, non-hemolytic 36, hemolytic.
C	2					95 " " non-hemolytic.	26, non-hemolytic 26 A, "
D	2 (incubator).	8.0	7.9	4.7	3,000,000,000	90 " " "	45, "
E	2 (room).	8 2	7.6	4.9	500,000,000	50 " " "	43, "

It will be noted from the table that Sample A was milk of high quality. It soured slowly, but between the 24th and 48th hours there appeared in the plates enormous numbers of non-hemolytic streptococci. These organisms practically overwhelmed all others and persisted throughout the observation. The hemolytic (mastitis) streptococci which were in the unsoured sample persisted until the 4th day. The non-hemolytic udder type was still found after 3 days of souring. Perhaps both types survived throughout, but could not be detected on account of the great number of other organisms. Sample B reveals about the same condition. The coagulation was more rapid and due to the growth of enormous numbers of non-hemolytic streptococci. The hemolytic streptococci observed in the fresh sample persisted for the 7 days allotted to the observation. They were unable to multiply with the same facility as the others. In Samples C, D, and E the non-hemolytic organisms also predominated.

It soon became apparent that souring could not be attributed to the common type of streptococci met with in udder and bottled milk. The udder types were able to persist through at least part of the souring period but not in sufficient numbers to be the cause of the phenomenon.

The detailed fermentative characters of the various strains noted in the protocols are given in Table II. It must be recognized that these cultures are merely types. Others have been studied but fail to show further differences from those recorded.

The figures given in Tables II and III represent the total acidity reached in the various tubes. All the fermented bouillon was of the same lot. Duplicate series were inoculated and incubated at 38°C. and at room temperature. The recorded figures represent the maximum acid production at the temperature best adapted for growth. Thus, Cultures 1, 3, 4 A, 7, 13, 18 A, and 20 grew better and produced more acid at 38°C., therefore the acid produced by them at this temperature has been recorded. On the other hand, all the other organisms adapted themselves better to temperatures of 20–22°C., so that the acid production has been given after incubation at this temperature.

TABLE II.

Fermentative Characters of Streptococci Noted in Table I.

Culture No.	Production of acid in.								Hemolysis
	Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose	Inulin.	Mannitol	Salicin.	
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
1	5.6	4.7	4.7	5.2	1.0	1.0	1.0	1.0	+
3	3.8	3.3	2.6	3.6	1.1	1.0	1.1	1.0	+
4 A	5.1	4.1	5.6	4.8	1.1	1.0	1.1	3.6	−
7	5.0	4.2	4.7	4.6	1.0	1.0	1.0	1.0	+
13	5.6	4.9	4.6	5 2	1.1	1.1	1.1	1.1	+
18 A	4.9	4.4	4.5	4 4	1.1	1.1	1.1	2.3	−
20	4.6	4.4	4.7	3.6	1.0	1.1	1.1	1.0	+
18	6.9	6 0	1.1	6 9	1.1	1.0	4.6	4.4	−
21	6.7	3.8	1.1	5.8	1.0	1.0	5.8	5.8	−
25 A	6.7	5.4	1.1	6 1	1.1	1.1	4 8	4.6	−
26	6.6	5.8	1.1	5.2	1.1	1.0	4.2	4.0	−
26 A	6 8	5 4	1.1	6 6	1.1	1.1	5.9	4 5	−
30	6.9	5 9	1 1	6.5	1.1	1.1	4.8	5.0	−
31	6.7	5.7	1.1	5.9	1.1	1.1	3.5	3 8	−
32 A	6.6	5.8	1.1	6.1	1.0	1.1	6 5	6.5	−
33	6.7	6.1	1.1	5 6	1.1	1.1	4.1	5.0	−
35	6.8	6.2	1.1	5 8	1.1	1.0	3.6	5 3	−
43	6.5	6.1	1.1	6.0	1.1	1.1	4.4	5 0	−
45	6.8	6.1	1.1	5.9	1.1	1.1	4 9	5.0	−
15	6 5	6.1	6.4	5.4	1 0	1.1	4.8	4 8	−
16	7.1	5.7	5.3	4.7	1.1	1.1	3 9	4.6	−
23	6.8	6.2	6.4	5.6	1.0	1.1	4 3	5.8	−
29 A	6.1	5.0	5.4	4.0	1.1	1.1	3.4	5.0	−
21 A	6.5	4.8	6.1	6.5	1.1	1.1	1 2	6 5	−
25	7.0	6.4	6.6	5.8	1.0	1.1	1.1	5.8	−
30 A	6.0	4.4	4.9	5.2	1.0	1.1	1.1	5.4	−
27	6.6	5.0	1.1	5.1	1.0	1.1	1.0	5.4	+
28	6.7	5.2	1.1	5.3	1.1	1 0	1.0	5.4	+
34	7.0	5.2	1.1	5.2	1.0	1.0	1.0	6.0	+
36	6.5	5.0	1.1	5.0	1.1	1.0	1.1	5.4	+

It will be noted that Cultures 1, 7, 13, and 20 are hemolytic streptococci which were found in both fresh and sour milk. These strains are identical with those associated with mastitis. They are aggluti-

nated by their specific group serum.   Culture 3 is similar to the low acid-producing streptococci found in the udder and in market milk. This species has been discussed in detail in a previous communication.[12] Cultures 4 A and 18 A are characteristic non-hemolytic mastitis streptococci which agglutinate with their specific antiserum.   All these cultures promptly coagulate milk at 38°C.   Litmus was not, as a rule, reduced even after coagulation.   Where reduction occurred it was usually confined to the bottom of the tube.   Since these well recognized udder and mastitis streptococci were unable to multiply to any great degree in soured milk, it is assumed that such is not their function.   In other words, their true habitat is within the udder.

In contradistinction to the preceding group, a second still' larger series, comprising Strains 18, 21, 25 A, 26, 26 A, 30, 31, 32 A, 33, 35, 43, and 45, made up the bulk of the flora of clotted milk.   These organisms differed in morphology from the mastitis types.   In bouillon the elements were usually elongated and arranged in pairs and short chains.   Spherical forms were also observed.   The mastitis cultures produced chains of cocci.   The second group of organisms attacked dextrose, lactose, maltose, mannitol, and salicin, but failed to ferment saccharose, raffinose, and inulin.   They produced more acid in dextrose than did the mastitis streptococci.   Litmus was reduced before milk containing it was coagulated.

The next four strains (Nos. 15, 16, 23, and 29 A) were isolated from two samples only.   They, too, differed from the mastitis streptococci morphologically.   They produced large amounts of acid in media containing dextrose, lactose, saccharose, maltose, mannitol, and salicin.   Litmus was reduced.

Cultures 21 A, 25, and 30 A also produced large amounts of acid. They attacked the same substances as the non-hemolytic mastitis streptococci but produced more acid.   Litmus was reduced by these cultures.   The characteristic rod-like and coccoid elements occurring in pairs and short chains are a further point in their differentiation. They grew best and produced a maximum acidity at room temperature. In addition they were not agglutinated by mastitis streptococcus serum.

[12] Jones, F. S., *J. Exp. Med.*, 1920, xxxi, 347.

The hemolytic streptococci (Nos. 27, 28, 34, and 36) which persisted throughout the souring of Sample B differed from the usual udder streptococci in several characters. Morphologically they

TABLE III.

Comparative Acid Production by Streptococci in 1 Per Cent Fermented Broth at Incubator and Room Temperature.

Culture No.	Incubator temperature.		Room temperature.	
	Titration.	Hydrogen ion concentration.	Titration.	Hydrogen ion concentration.
	per cent	*pH*	*per cent*	*pH*
1	5.6	4.8	5.0	4.9
4 A	5.3	4.9	4.6	4.9
7	5.0	4.7	5.1	4.9
13	5.8	4.8	4.8	4.8
18 A	4.9	4.7	4.4	4.9
20	5.6	4.7	4.8	4.9
18	6.0	4.7	6.9	4.6
21	5.8	4.8	6.3	4.4
25 A	5.3	4.7	6.7	4.5
26	5.0	4.8	6.6	4.6
26 A	5.4	4.7	6.8	4.6
30	5.9	4 8	6.9	4.6
31	5.2	4.8	6.7	4.6
32 A	5.1	4.9	6.6	4.6
33	5.4	5.0	6.7	4.5
35	5.6	4.9	7.0	4.5
43	6 0	4.6	6.5	4.5
45	5.7	4.8	7.0	4.4
15	5.8	4.8	6.9	4.6
16	7 0	4.6	7.1	4.5
23	5.6	4.9	6.8	4.5
29 A	4.4	4.9	6.1	4.5
21 A	6.0	4 7	6.5	4.5
25	5.9	4.7	7.0	4.5
30 A	4.4	4.8	6.0	4.6
27	7.0	4 5	6.9	4.5
28	7.0	4.7	6.7	4.5
34	7.1	4.9	6.9	4.5
36	7.1	4.7	7.0	4.4

resembled the sour milk types. They did not reduce litmus completely and failed to ferment saccharose. They grew as well at 38°C. as at room temperature.

It has been asserted that the sour milk organisms grew better and produced more acid at room temperature. Experimental evidence that this is the case is offered in Table III. Duplicate inoculations into fermented bouillon of the same lot containing 1 per cent dextrose were made; one set was incubated at 38°C., the other at room temperature. Titrations and hydrogen ion concentration readings were made after 7 days. All cultures grew well at both temperatures. The results are given in Table III.

The figures indicate that cultures of the mastitis type (Nos. 1, 4 A, 7, 13, 18 A, and 20) produced more acid when grown in the incubator. The reverse is true for the others except for the hemolytic strains (Nos. 27, 28, 34, and 36). That the true milk-souring streptococci grow better at ordinary temperatures (20–22°C.) certainly points to other sources of origin than the saliva, feces, or udder of cows. Cultures 27, 28, 34, and 36 differed from both groups in many characters. They may be saprophytic forms which have gained entrance into the udder and are still adapting themselves to a new environment.

The pathogenic properties of the sour milk streptococci have been studied by so many that further experimental evidence seemed unnecessary. Many have reported the entire lack of pathogenesis of the members of this group for laboratory animals. Unfortunately such findings cannot be regarded as a method of differentiation between mastitis and sour milk streptococci, since the mastitis streptococci are also lacking in virulence for species other than bovines.

DISCUSSION.

From the preceding observations it seems clearly established that the lactic acid group consists of at least three species of organisms. Each species possesses characters which differentiate it from the streptococci which are associated with mastitis and the low acid-producing udder forms.

Sufficient data to warrant the assertion that *Streptococcus lacticus* is an udder inhabitant have not been brought forth. Sherman and Albus first showed conclusively that there existed distinct group differences between the udder and the milk-souring streptococci. Aside from the specific cultural differences here reported a number of other points throw light on the question. The milk-souring types

have not been met with in milk drawn directly from the udder. Such organisms have not been found in the fresh bottled milk from a dairy where a great many examinations of udder milk have been made. Mastitis and udder streptococci are usually present in this bottled milk. The milk-souring group grows best and produces more acid at a temperature of 20–22°C. The udder streptococci prefer a higher temperature (38°C.).

Rogers and Dahlberg[18] found in milk in a few instances non-saccharose-fermenting streptococci which attack mannitol. They also studied over 50 strains of udder origin. Among these but one attacked mannitol without acidulating media containing saccharose. This organism liquefied gelatin.

Many have pointed to several possible sources of origin. The saliva, feces, and skin of cows have been mentioned particularly. The specific identifications were usually made on morphological findings. Many of the streptococci from the saliva, skin, and feces do resemble the lactic acid organisms in form, but they differ in other essentials. The writer has isolated 35 strains of streptococci from the saliva of cows. Although mannitol fermentation occurred in one-third of the cultures, every strain fermented saccharose. In addition the amount of titratable acid produced in dextrose rarely exceeded 4 per cent. The bovine fecal streptococci were characterized by their ability to produce large amounts of acid in dextrose, lactose, saccharose, maltose, raffinose, inulin, and salicin. Mannitol was not fermented. Streptococci from the skin produced acid in fermented broth containing raffinose, saccharose, and mannitol in addition to dextrose, lactose, maltose, and salicin. One feels justified in asserting that if *Streptococcus lacticus* inhabits these regions it exists in such small numbers that it cannot be detected. The souring of milk cannot, therefore, be attributed to the usual types found in the saliva, feces, or vagina or on the skin of cows.

Sufficient evidence exists to rule out the udder or mastitis group as the usual cause of souring under natural conditions. These organisms are readily detected in bottled milk, but cannot be found in plate cultures prepared from milk soured for 3 or 4 days. When

[18] Rogers, L. A., and Dahlberg, A. O., *J. Agric. Research*, 1913–14, i, 491.

equal amounts of cultures of lactic acid and udder streptococci were added to tubes of sterile milk, the udder streptococci multiplied for 24 hours. Plates prepared from such milk after 48 hours at room or incubator temperature reveal only the lactic acid type. Under natural conditions probably other bacteria so alter the environment that the udder group is able to survive but not to multiply to any considerable degree.

I am inclined to agree with Kruse and others that the lactic acid organisms resemble more closely the genus *Streptococcus* than the genus *Bacterium*. Their growth on solid media and their fermentation characters resemble those usually associated with streptococci. The name *Streptococcus lacticus* I is suggested for the largest group (Table II), characterized by the fermentation of dextrose, lactose, maltose, salicin, and mannitol and their inability to attack saccharose, raffinose, and inulin. *Streptococcus lacticus* II may be considered as differing from the former group in its ability to ferment saccharose in addition to dextrose, lactose, maltose, mannitol, and salicin.

SUMMARY.

A well defined group of rod-like and coccoid organisms arranged in pairs and chains has been encountered in sour milk. The group comprises at least three species; the largest number ferment dextrose, lactose, maltose, mannitol, and salicin, and fail to ferment saccharose, raffinose, and inulin. A smaller number ferment saccharose in addition to dextrose, lactose, maltose, mannitol, and salicin. A few fail to attack mannitol. All three types grow luxuriantly at room temperature, coagulate milk, reduce litmus, and produce large amounts of acid in fermented bouillon containing dextrose.

Specific morphological and cultural differences exist between the lactic acid streptococci and those associated with mastitis and those occurring in the udder. The lactic acid organisms outgrow the udder streptococci in the milk-souring process. When both types are implanted in sterile milk the udder type soon disappears.

THE FAT CONTENT OF FECES OF YOUNG CALVES.

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New-born calves excrete feces during the first week of life which show distinct variations in the gross appearance. A study was made, therefore, of the amount of fat present in the feces, from birth to the time when the calves were approximately a week old. It is during this time that the calf readjusts its metabolic activities from that of an intra-uterine existence to one in which it subsists by absorption from its gastro-intestinal tract. It was found in general that the fat content of feces increases from birth to approximately the third day after which it decreases and assumes an approximately normal level. At the time of the high fat content, there is present in the feces a greater proportion of fatty acids in the form of soap, indicating a lack of absorption on the part of the young animal.

Method.

The method used for the determination of fat was a modification of the method developed by Saxon,[1] which is in itself a development of the method of Meigs, for the determination of fat in feces. It seemed advisable to estimate the quantity of soap present in feces, as well as the total fat and fatty acids present as free fatty acids and soap. To do this we first extracted moist feces with ether and alcohol, according to the Saxon procedure, and then to the residue added hydrochloric acid to liberate the fatty acids from the soaps. The ether layer when removed contains a small amount of alcohol and acid; it was, therefore, collected in a separatory funnel and washed with distilled water until free from chlorids. This precaution is necessary to prevent the formation of ethyl esters of the fatty acids present.

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[1] Saxon, G. J.: J. Biol. Chem. **27**:99, 1914.

Comparative analyses showed this procedure to give, with suitable precautions, the same results as in the Saxon procedure.

After we had commenced our analyses, the modification of the Röse-Gottlieb method by Holt, Courtney and Fales[2] appeared. These investigators applied a somewhat similar procedure to dried feces, by extracting with combined ether and petroleum ether. They derived their value for soaps by subtracting the results obtained by a nonacid extraction from that obtained by an acid extraction. When such a procedure is applied to work with fresh feces we do not obtain results comparable with those obtained by a second extraction, i.e., fat is obtained through the second extraction which does not appear in the first extract.

From our experiences with this modified Saxon method for the determination of fat and for the distribution of neutral fat, fatty acids and soap in feces, we have found that the method gives consistent results for total fat. When used for the estimation of fatty acids and for the estimation of soap and free fatty acids as we have modified it, the method gives fairly satisfactory results. In feces of a high fat content, rich in soap, the double extraction tends to give low results for total fat, but the loss appears to be in neutral fat rather than in the fatty acids and soap. We had hoped to develop the double extraction method to such an extent that we could dispense with the acid extraction but found it necessary to continue our determination according to the Saxon procedure as well as our own as a check on the results. For the acid alcohol-ether extraction the method in detail is as follows:

From 5 to 6 grams of thoroughly mixed moist feces are weighed into a 100 c.c. glass stoppered cylinder, as suggested by Saxon. A small amount of water is added, and the cylinder is shaken to obtain a thorough mixture of feces and water. Then from 1 to 2 c.c. of concentrated hydrochloric acid is added and the volume made up to 30 c.c. Twenty cubic centimeters of ether and 20 c.c. of alcohol are added and the contents shaken for five minutes after each addition. After the last shaking the cylinder is allowed to stand until the ether-fat layer separates. The ether is blown off into a separatory funnel,

[2] Holt, L. E., Courtney, A. M., and Fales, H. L.: Am. J. Dis. Child. **17**:38 (Jan.) 1919.

containing a small amount of water. Four portions of ether of 5 c.c. each are added successively, the contents agitated, and the ether layer removed.

A second portion of 20 c.c. is then added and the contents shaken for five minutes. This layer is then removed and four 5 c.c. portions are again added and removed. The ether extract is thoroughly washed with water until free from chlorids, transferred to an Erlenmeyer flask and the ether removed by evaporation on a hot plate. The fat residue is then extracted with petroleum ether, boiling below 60 C., and the extraction filtered through a small fat-free filter paper, into a weighed flask. The petroleum ether is removed and the fats dried at approximately 90 C. to constant weight. Fifty cubic centimeters of benzol are added to the weighed fat, heated to boiling, and immediately titrated with tenth normal potassium alcoholate. This procedure gives the total fat and fatty acids, present as free fatty acids or soap.

Double Extraction.—The procedure in this case is practically identical with that just described with the following exceptions:

(a) Extraction without Acidification: In the first original extraction the hydrochloric acid is omitted, and the ether extract is passed through a fat-free filter to retain any particles of soap which may be carried over with the ether.

(b) Acidification and Extraction: The residue remaining from the extraction under (a), plus the filter paper from (a), is acidified with from 1 to 2 c.c. of hydrochloric acid and the extraction repeated as outlined. The results obtained under (a) above represent most of the fat and the free fatty acids. The extraction under (b) contains the residual fat, if any, and the fatty acids present as soaps.

We have found that it is necessary to work with fresh feces because when the feces are kept for any length of time there is a gradual transformation of fat into fatty acid as well as rearrangements in the fatty acid-soap equilibrium. One of the largest possible sources of error, as indicated by Holt, Courtney and Fales, is in mixing the feces to obtain a thoroughly representative sample for analysis.

Moisture was determined by weighing from 5 to 7 gm. of moist feces into a weighed lead cap and drying in a vacuum desiccator over sulphuric acid. The hydrogen ion concentration was determined

colorimetrically on a filtered water extract of the moist feces using the indicators and standard solutions of Clark and Lubs. Nitrogen was determined by the Kjeldahl method.

Subjects.

The animals studied were born at the Institute and kept in stalls separate from their mothers. They were taken to the dam to suckle twice a day, night and morning, or were fed milk from the dam from a pail.

Three calves did not receive colostrum but were given a mixed whole milk. With the exception of number 456B, which was a Guernsey, all animals were either grade or purebred Holsteins.

Collection of Feces.

The feces were collected in glass jars at the time of defecation. The collections were not quantitative. Analyses were begun soon after collection.

Data.

Data were obtained from fourteen calves, over periods ranging from four to twenty-four days for each animal. Additional preliminary data on samples of feces obtained from different calves at various ages show results similar to those presented as to the sequence of changes in the same calf.

The data are contained in Tables 1 and 2. The results for fat, fatty acids and soap are expressed as percentages, in terms of the moisture-free substance and the nitrogen as percentage of the moisture- and fat-free substance.

The data show an increase in the fat content from birth to approximately the third day, after which the fat content decreases to a somewhat lower and fairly uniform value. Associated with a high fat content on the third day is noted an increased percentage of soaps.

The physical character of the feces corresponds somewhat to the variation in fat content, the meconium is usually greenish black in color; it may consist of hard balls, or it may be soft and rich in mucus; quite often the latter type of stool follows the former type. Succeeding the meconium are feces yellow in color and of a more or less

TABLE 1.

Composition of the Feces of Young Calves.

(Fat percentage of the dry sample. Nitrogen as percentage of the moisture- and fat-free sample.)

No.	Age, Days.	Solids, per Cent.	Total Fat. per Cent.	Neutral Fat, per Cent	Fatty Acids as Soap, per Cent.	Free Fatty Acids, per Cent.	N per Cent.	pH
657	½	38.4	11 0	9.8	0.1	1.1		
	1	36.0	14 4	13.4	0.1	0.9		
	3	27.0	15.1	7.7	0.9	6.4		
	4	29.7	14.8	8.6	0.9	4.4		
	5	24.5	9.6	7.0	0.4	2.2		
336B	½	40.9	20.1	18.8	0.2	1.2	7.0	
	2 a.m.	39.6	10.5	6.6	1.0	2.9	11.9	
	2 p.m.	38.3	11.5	6 2	2.1	3.2	12.3	
	3	27.3	6.2	4.4	0.0	1.7	12.6	
	4	27.5	7.0	4.1	0.0	2.9	11.6	
479B	½	43.7	15.1	13.7	0.5	1.0		
	1	40.7	9.1	2.8	4.1	2.2	12.5	
	3	34.1	26.1	17.9	1.4	6.8	12.3	
	6	29.3	7.4	3.6	0.2	3.5	11.6	
489B	1	35.1	16.3	15.4	0.3	0.5	7.6	
	2	40.4	11.9	6.3	2.0	3.6	9.9	
	4	28.6	10.6	7.1	0.2	3.4	12.0	
	7	31.3	9.3	4.1	0.2	5.1	9.5	
	9	29.6	5.8	3.3	0.2	2.2	9.0	
488B	1	44.9	3.4	1.6	0.2	1.7	13.6	
	2	43.0	4.1	1.5	0.2	2.4	13.7	
	4	35.9	12.3	5.2	0.5	6.6	12.0	
	5	43.6	14.6	9.1	0.6	4.9	9.6	
	12	21.9	13.9	7.6	0.4	5.9	8.1	
	13	28.1	43.7	23.1	4.4	16.2	7.8	
	20	31.3	21.7	2.8	13.8	5.1	8.3	
759	1	26.5	6.6	6.1	0.1	0.4	....	6.6
	2	36.3	24.9	14.0	6.6	4.4	....	6.2
	3	36.7	16.3	7.6	1.8	6.9	....	5.8
	4	37.1	11.2	4.3	0.3	6.6	....	6.2
	6	26 0	17.2	8.5	0.9	7.6	....	6.2
	7	28.4	13.9	4.9	0.2	8.8	....	6.2
756	2	38.7	20.2	12.6	1.2	6.4	....	6.2
	3	33.1	24.5	4.4	14.0	6.0	....	6.1
	4	33.1	7.8	2.4	0.1	5.4	....	5.6
	6	45.5	7.9	4.7	0.9	2.4	....	5.6
	7	43.4	10.3	6.4	2.2	1.8	....	5.7
	9	33.6	8.5	3.3	0.5	4.7	....	5.6

TABLE 1.—*Concluded.*

No.	Age, Days	Solids, per Cent.	Total Fat, per Cent.	Neutral Fat, per Cent.	Fatty Acids as Soap, per Cent.	Free Fatty Acids, per Cent.	N, per Cent.	pH
465B	1	39.7	14.5	12.9	0.3	1.3	7.3	6.7
	2½	33.9	24.0	14.5	3.7	5.7	9.0	6.2
	3½	33.9	37.5	21.1	4.7	11.7	9.5	5.7
	4½	21.8	10.6	5.6	0.0	3.9	10.1	5.7
	6½	33.3	10.8	5.0	0.5	5.2	11.6	5.6
478B	1	43.4	16.6	14.0	0 3	2.3	....	6.9
	2	34.2	33.0	17.8	7.2	8.1	13.4	5.9
	3	32.7	26.4	12.7	5.5	8.1	13.1	5.4
	8	24.5	16.4	10.3	0.6	5.5	13.1	5.6
480B	1	41.2	3.2	1.3	0.0	1.9	20.2	6.4
	2	46.1	7.7	3.0	1.3	3.4	11.4	5.8
	3	38.1	20.7	15.3	2.2	3 3	11.8	5.9
	4 a.m.	33.1	15.5	10.2	1.5	3.8	11.2	6.1
	4 p.m.	34.7	12.8	9.4	0.6	2.8	11.9	
	5	42 0	14.1	7.8	0.5	3.8	9.3	
477B	1	23 9	16.6	10.3	1.2	5.1	12.6	5.9
	2	29.8	19.7	13.4	1.7	4.6	10.0	5.6
	2	37.7	9.2	4 0	1 4	3.8	12.8	5.6
	3	40.7	27.0	19.6	0 7	7.2	10.2	5.8
	7	30.3	19.8	10.7	0 2	8.9	10.0	5.2

Calves Which Did Not Receive Colostrum

No.	Age, Days	Solids, per Cent.	Total Fat, per Cent.	Neutral Fat, per Cent.	Fatty Acids as Soap, per Cent.	Free Fatty Acids, per Cent.	N, per Cent.	pH
478B	1	40.5	8.7	7.1	0.6	1.1	8.4	
	5	32.1	15.3	5.9	0.3	9.1	8.7	6.8
	6	26.3	51.4	12.4	21 9	16.8	7.5	5.2
	7	26 5	47.3	7.8	21.1	20.2	7.1	6.2
	8	26.6	42.7	4.4	17.5	20.8	6.4	6.4
	11	29.7	22.3	6.6	9.2	6.4	8.2	6.8
	12	28.2	11.7	6.2	1.0	4.4	10.3	6.8
	15	31.7	8.9	6.7	0.0	2.3	8.3	6.6
495B	1	32.5	8.6	7.9	0.3	0.3	6.9	6.8
	7	31.5	16.4	4.6	3.1	8.7	9.9	5.8
	8–9	33.7	18.0	6.3	4.2	8.8	10.4	6.2
	10	28.4	12.8	4.7	3.9	4.3	10.8	
	13	36.2	11.4	5.7	2.3	3.4	8.9	7.2
474B	1	35.6	16 1	14.8	0.3	0.9	7.7	6.8
	5	28.6	13.1	7.1	0.6	5.5	11.3	5.4
	6	25.8	5.6	2.8	0.8	2.2	9.7	6.2
	7	32.4	6.3	3.1	0.0	3.2	9.0	5.4
	8	22.0	9.1	5.3	0.0	3.8	9.0	5.8

pasty consistency, often containing fatty acid crystals. This is the stool most characteristic of approximately the third day. Following this is the fairly soft stool, greenish brown in color, the type of feces which persists unless a digestive disturbance occurs. The separation between the two types of feces was very marked—when the two types appeared in the stool they could, as a rule, be differentiated readily.

The fat-soap mixture remaining after titration of the fatty acid was examined for nonsaponifiable material. From 6.1 gm. of fat, fatty acids and nonsaponifiable matter 0.17 gm. of nonsaponifiable material was obtained representing approximately 2.8 per cent. of the

TABLE 2.

Average Composition of the Feces of Young Calves with Relation to Age.

Age, Days	Number of Analyses Represented in Average	Solids, per Cent.	Total Fat, per Cent	Neutral Fat, per Cent	Fatty Acids as Soap, per Cent.	Free Fatty Acids, per Cent.	N, per Cent	pH
1	13	37.6	13.8	10 6	1.1	2.1	11.8	6.4
2	10	38.4	14.4	8.2	2.1	4.0	9.1	5.8
3	9	33.7	22.2	12 3	3.5	6.4	11.5	5.7
4	9	31 3	11.4	6 3	0.4	4.4	11.4	5.9
5	3	36.7	12 7	7.9	0 5	3 6	9.4	
6	4	33.5	10.8	5.4	0 6	4.6	11 6	5.8
7	4	33 3	13 3	6 5	0 7	6 1	9 7	5.7

mixture. This value is undoubtedly high. The nonsaponifiable material was largely cholesterol.

During the time that calves are showing the change in the appearance and composition of feces they are receiving a variable diet. The first milk or colostrum is rich in protein and the colostral bodies but is not, according to published analyses,[3] appreciably richer in fat than milk secreted after lactation has proceeded for some time. The colostral bodies do carry the fat in the form of larger droplets than occur in milk.

It seemed possible that the colostrum itself might be responsible for the character and composition of feces passed during the first

3. Eckles, C. H., and Palmer, L. S.: Research Bull. 25, Missouri Agric. Exper. Sta., 1916; König., Chem. Nährungs u. Genussmittel, 1: 1903.

three days. Three calves, 487 B, 495 B, 474 B were, therefore, fed the whole mixed milk from cows well along in the period of lactation. In all cases the meconium was passed readily, but subsequent defecation was delayed. Calf 487 B passed meconium on the first day and on the second day a sample consisting largely of meconium with a small amount of yellow pasty feces. A sample was not obtained, nor so far as we could determine was passed, between the second and fifth days. On the fifth day three stools, small in amount, were passed of approximately the same consistency. An analysis was obtained, on the third sample, the others being unsatisfactory for analysis. These samples were yellow, pasty in consistency similar to those obtained immediately after the meconium. This type of stool was then followed by daily passages of feces, high in fat content and white in color, apparently an abnormal product. It was not until the eleventh day that the character of the feces returned approximately to normal. The other calves, 495 B and 474 B, likewise showed delay of defecation after the passage of meconium. The second sample passed by calf 495 B was obtained on the fourth day after birth but was dropped in the bedding and could not be analyzed. The greenish brown stool ordinarily passed about the fourth day did not appear until the eighth or ninth day. With calf 474 B the second defecation occurred on the fifth day and greenish brown feces appeared on the seventh day. The chemical composition of the feces of these last two calves, if they be considered in the sequence of defecation rather than the actual date after birth on which they were passed, follows somewhat the sequence of changes characteristic of normal feces. We do not feel, therefore, that the increased fat content and high soap is necessarily related to the colostrum.

In addition to the data recorded, tests were made on a filtered water extract of the feces for the presence or absence of coagulable protein. In practically all cases we found protein present which was coagulable with heat and a small amount of precipitate in cold with acetic acid.

In certain cases in which the coagulable protein was particularly rich, this protein was extracted and examined as to its character, as follows: Feces were worked up with distilled water, acidified with acetic acid, centrifugalized, the protein precipitated with ammonium

sulphate, dialyzed and reprecipitated at least three times. Other precipitations were made with saturated sodium chlorid. Two globulins were obtained, one having precipitation limits for ammonium sulphate between 2.8 and 3.4 and precipitated by dialysis, and the other having precipitation limits between 3.4 and 4.4, and not precipitated on dialysis. Both proteins were coagulable with heat. In addition protein was obtained on complete saturation with ammonium sulphate coagulable with heat.

Soap in Feces.

Feces obtained during the second to the fourth day were found by our analysis to be rich in soaps, had a peculiar glistening appearance, and when a small portion of such material was suspended in water, in which it was insoluble, it gave an effect such as occurs when finely divided metal is suspended in gasoline. It was also noticed in the course of the nonacid extraction that white particles tend to accumulate between the aqueous layer and the ether-fat mixture.

It appeared possible that we might be able to separate this material, which we assumed to be and subsequently found to be soap, from the associated material. Portions of fecal material were, therefore, subjected to the procedure adopted for the extraction of fat and free fatty acids, i.e., to nonacid extraction, except that this material was kept suspended in the ether layer by agitation and siphoned off before it could settle. The material obtained was then thoroughly extracted with ether in a separatory funnel. When suspended in a 50 per cent. alcohol-water mixture, by varying the proportions of alcohol and ether, we were able to remove fecal material of greater specific gravity than the soap which had been carried over in the original separation. As a result we obtained a product which was practically pure white, but which on decomposition was found to carry a small amount of yellow pigment which was transformed in an acid solution to a green pigment, evidently a bile pigment. These crystals were thoroughly washed with ether, dried and again extracted with ether until no more extractable material could be obtained. The following results obtained on analysis showed the soap to be chiefly calcium stearate.

TABLE 3.

Analysis of Stool for Soap.

Weight of sample....................................	0.5849 gm.
Weight of fatty acids................................	0.5216 gm.
Calcium present....................................	0.0369 gm.
Magnesium	Trace
Titration of fatty acids with 0.1144 N. potassium alcoholate....	16.34 C.c.
Molecular weight from calcium determination...............	283
Molecular weight from fatty acid titration.................	279
Molecular weight of stearic acid.......................	284
Iodin value..	3.9
Stearic acid (Hehner and Mitchell).....................	86%

DISCUSSION.

The changes in composition during the first week of the life of calves indicate that there is a readjustment in the metabolic activities of the calf during the first three days. The data obtained suggest a fairly complete digestion of fat, but a partial failure to absorb it. whether or not the failure to absorb the fatty acid is due to a specific condition of the intestinal mucosa or to physical or chemical conditions which influence the combination of the fatty acids with calcium has not been determined. It seemed possible that colostrum might in some way be related to this increased excretion of fat and the excretion of soaps. The effect of colostrum was to delay the excretion of fecal material. Examination of the data in relation to the sequence of defecation without reference to time, in the two calves which may be considered as normal, indicate a somewhat higher fat content of the feces immediately following the meconium.

That there are metabolic disturbances during the first days of life, or earlier, are indicated in the work of various authors. Theobald Smith (unpublished notes) has noted in some cases, the presence of protein in the urine of new-born calves, which disappears on approximately the third day. Reusin[4] and Schloss and Crawford[5] have noted a high uric acid excretion in new-born infants which reaches its maximum on approximately the third day. Kingsbury and Sedg-

4. Reusin, H. Z.: Geburtsh. u. Gvnäk. 32:36, 1895.
5. Schloss, O. M., and Crawford, J. L.: Am. J. Dis. Child. 1:203 (Feb.) 1911.

wick[6] have noted a similar phenomenon for the blood of new-born infants. Scours occurs in new-born calves ordinarily within the first three days. It seems to us that the increased fat content of the feces of new-born calves on the third day is probably associated with the other readjustments which are taking place in the animal. It is interesting that in a change of diet such as from fasting to a normal diet, metabolic activity is readjusted to the new plane in approximately three days.[7]

CONCLUSIONS.

1. Data have been presented showing the presence of a high fat content on approximately the third day in the feces of new-born calves. This high fat content is accompanied by a relatively high percentage of soap.

2. Calves which did not receive colostrum passed the meconium readily but showed a delayed defecation subsequently.

3. From feces of high soap content nearly pure calcium stearate was separated.

6. Kingsbury and Sedgwick: J. Biol. Chem. 31:261, 1917.
7. Howe, Mattill and Hawk: J. Am. Chem. Soc. 33:568, 1911.

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